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RELATION BETWEEN OBESITY AND BLOOD PRESSURE IN CHILDREN: POSSIBLE ROLE OF POLYUNSATURATED FATTY ACIDS AND THEIR METABOLITES VIA CYTOCHROME P450

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Abstract

Background: An unbalanced diet and a sedentary lifestyle are the main determinants of obesity and the related metabolic and cardiovascular (CV) diseases. Quality of dietary fat, beyond the quantity, can influence CV risk profile and in particular omega-3 fatty acids (PUFA) have been proposed as beneficial in this setting. The aim of the first study was to evaluate the associations between erythrocyte membrane FA, markers of average intake, and individual CV risk factors, characterizing the metabolic syndrome (MetS), in a group of 70 obese children**.** In the second study we focused especially on omega-3 and omega-6 PUFA and their metabolites via CYP450/soluble Epoxide Hydrolase (sEH) in relation to blood pressure (BP) and markers of vascular function in the same sample of obese children. The aim of the third school-based study was to assess the association between body fat distribution, BP and vascular function also in relation to erythrocytes membranes PUFA in 309 children aged 8-10 years.
Results: Omega-3 Index was low (about 4%) in all children included in the studies. In study 1, we found that omega-6 PUFA, especially arachidonic acid (AA), were inversely related to most of the characteristics of the MetS: waist circumference, triglycerides, insulin, SBP and Fatty Liver Index (FLI), whereas saturated FA (SFA) and in particular palmitic acid (PA) were directly related to the same features. In the second study, AA was inversely correlated with SBP and DBP in the whole population and in normotensive (n: 53/68), whereas in hypertensive linoleic acid (LA) was inversely related to SBP and DBP. Omega-3 PUFA were inversely related to the Reflection Index (RI), an indirect marker of relative vasoconstriction, whereas eicosapentaenoic acid (EPA) to the z-score of carotid intima-media thickness (cIMT). DiHOMEs, metabolites of LA via cytochrome P450/soluble Epoxide Hydrolase (CYP450/sEH), showed a direct correlation with office-DBP and nighttime-DBP in the whole sample, but especially in normotensives. Only in the hypertensive subgroup, epoxyeicosatetraenoic acids (EEQs), metabolites derived from EPA, and epoxydocosapentaenoic acids (EDPs), derived from docosahexaenoic acid (DHA), were inversely correlated to BP and vascular structure. In the third study, we found a high prevalence of overweight and obesity (19% and 13%, respectively). BP was directly related to several anthropometric characteristics and indexes of central adiposity showed stronger association in obese children than in normal weight subjects. Obese children had higher BP and z-score of PWV in comparison to normal-weight children. Omega-6 PUFA inversely correlated with triglycerides, whereas omega-3 PUFA with glucose and triglycerides.
Conclusions: Taken together, our data show a high impact of weight excess on BP homeostasis and on arterial stiffness, detectable also in early childhood. Omega-6 PUFA could exert a beneficial effect on most of the features of MetS, at least in obese, whereas the role of omega-3 PUFA, whose assumption appeared very low, and of their metabolites via CYP450 resulted of minor importance or confined to specific subgroups. Further investigations are needed to better clarify the role of omega-6/omega-3 PUFA and their derived metabolites via CYP450/sEH in BP homeostasis and MetS in children as well as in adults.

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Introduction

Definition and prevalence of obesity in children

Since the last 90s, the Word Health Organization (WHO) has started to claim that overweight and obesity were representing an increasing epidemics not only in adults but even in children. Since the 70s, the prevalence of obesity and its associated comorbidities, including type 2 diabetes, has been rising[1]. Anyhow, the different definitions of obesity and different national reference values make it difficult to quantify the real prevalence and incidence of obesity worldwide[2]. In adults, the cut-off points for the definition of overweight and obesity, namely a body mass index (BMI) higher than 25 and 30 kg/m^2 respectively, are generally accepted[3]. In children, BMI changes with age and therefore the International Obesity Task force (IOTF) suggested to link the adult cut-off points to the BMI centiles for children, thus identifying specific values for each age and sex[4].
To date two international datasets are used to define overweight and obesity in children: the IOTF reference and the WHO standards, which present some discrepancies due to the different populations and the choice of cut-off points. Some comparison studies have shown that WHO standards tend to overestimate the body weight excess as compared to IOTF reference[5].
According to current guidelines, the 85th percentile of BMI is the threshold for overweight and obesity is defined over the 95th percentile[6,7].
Regarding adulthood, it has been estimated that the prevalence of obesity in 2014 was 10.8% in men and 14.9% in women and severe obesity (i.e. BMI> 35 Kg/m^2) was 2.3% and 5.0%, respectively[8].
Large surveys have collected data for children aged 0 to 5 years old, whereas the studies in children older than 5 years and in adolescents are smaller. The available data indicate that in the last three decades the prevalence of obesity has risen not only in industrialized Countries but also in low and middle-income Countries.
In 2004, the prevalence of overweight and obesity worldwide was 10% and 2-3%, respectively[9].
In the U.S.A. the prevalence of weight excess in school-aged children in 2003–2004 was 35% and obesity accounted for 13%[9]. In eight European countries between 2007 and 2010, 18,745 children participated in the IDEFICS Study that reported a combined prevalence of overweight and obesity of more than 40% in southern Europe and less than 10% in Northern Countries. The prevalence of overweight was, overall, higher in girls (21.1%) than in boys (18.6%)[10].
In Italy, the data collected in 8-9 years-old children from the national surveillance system "Okkio alla salute" reported a prevalence of overweight and obesity of 23.3% and 12.0%, respectively, in 2008-2009, which slightly decreased to 22.2% and 10.6%, in 2012. As in Europe, even in Italy a North-South gradient was detectable, showing higher prevalence of weight excess in southern regions. The estimated prevalence of severe obesity in 2010 in children aged 8-9 year-old was 4.5% according to the WHO definition and 2.7% with IOTF reference[11].
A few studies investigated the prevalence of obesity in school children.
In a Chinese sample of 88,974 adolescents from 49 middle schools, 14.6% of male and 8.6% of girls were overweight, 7.0% of boys and 2.9% of girls were obese, according to Chinese reference data. The prevalence of pre-hypertension and hypertension was 7.2% and 3.1%, respectively[12]. Similar results of

overweight/obesity prevalence were found also in another large school-based study in Shanghai, China[13]. A large multicentric study reported the prevalence of overweight in school-aged children of 13 European countries, which ranged from 7% to 23% across the different countries[14]. In Italy a cross-sectional survey of childhood obesity was performed on 3923 children aged 6-11 years from 19 schools. 26.7% of boys and 25.2% of girls were overweight, 21.1% of boys and 20.7% of girls were obese. The prevalence of hypertension was 9.9% in boys and 13.9% in girls[15].

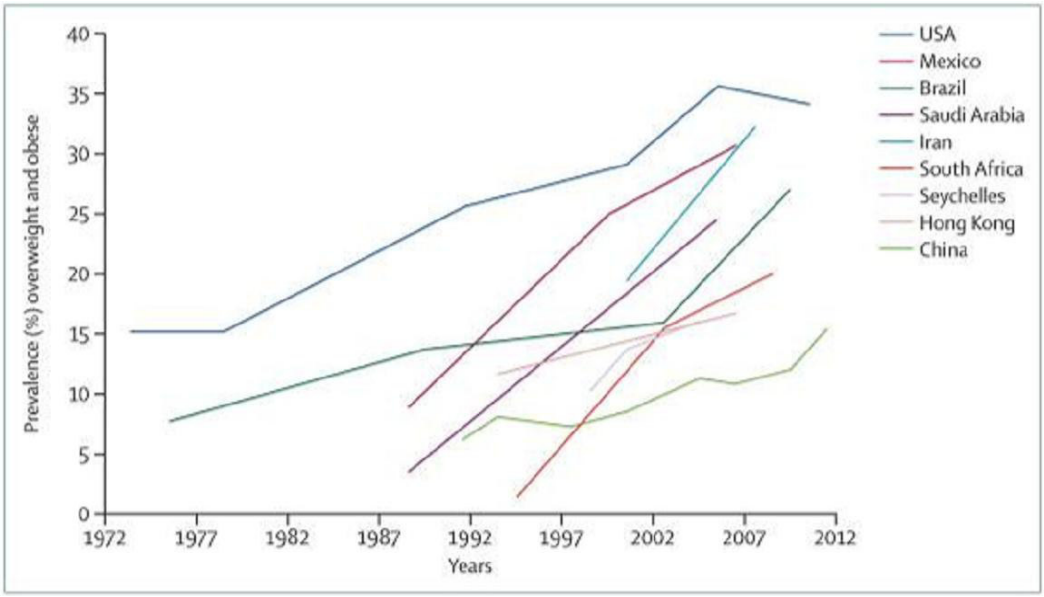


Figure 1. Reproduced from Lobstein T et al., *Lancet* 2015[16]

Trend in obesity and overweight prevalence in USA and in 8 low-income and middle –income countries

Comorbidities associated to obesity

Obesity is associated with a wide range of metabolic and hemodynamic alterations in children as well as in adults. Atherosclerotic process begins in early childhood, as documented in autoptic studies that identified atherosclerotic lesions in the aorta and in coronary artery in 3 years-old children[17] and the extent of the lesions was associated with obesity degree and others cardiovascular risk factors[18].

Previous studies in obese children have documented an impairment of vascular function tests, like an increase in intima-media thickness, impairment of endothelial function, higher arterial stiffness[19,20] leading to higher blood pressure levels[21]. Some data suggest that weight excess during childhood is associated with dyslipidemia in adulthood[19]. Moreover, obesity in adolescents is associated with glucose intolerance and insulin resistance[22,23]. Obesity, and

in particular central distribution of the fat, is associated with higher cardiovascular risk profile through the clustering of CV risk factors that lead to the metabolic syndrome. Non-alcoholic fatty liver disease (NAFLD) can be considered the hepatic manifestation of MetS because of the common risk factors and in particular insulin sensitivity. MetS in adults has been defined as the cluster of abdominal obesity, high triglycerides and low HDL-cholesterol levels, high blood pressure and type 2 diabetes or impaired fasting glucose, whose cut-off points are clearly stated[24]. In children, there is not yet a consistent set of cut-off points and the definition of MetS is still under evaluation.

Hypertension in children and adolescents

Elevated blood pressure is often detected in childhood due to the raise in the incidence of obesity and to the major attention on the detection of this condition also in children. Indeed, longitudinal studies demonstrated that children and adolescent with elevated blood pressure often become hypertensive adults[25]. Diagnostic criteria for hypertension in children are based on the influence of age and anthropometric characteristics on blood pressure, thus needing individualized cut-off points for the different ages and growth development. The current European guidelines suggest the adoption of normative values based on data collected from more than 70,000 children of United States using auscultatory method, with cut-off calculated for age, sex and seven height percentile[26].

Hypertension in children aged 1 to 16 years is defined as SBP and/or DBP persistently at least 95^{th} percentile measured in three separate occasions, whereas high-normal BP is defined for SBP and/or DBP between 90^{th} and 95^{th} percentile. For older adolescents instead, current European guidelines suggest to use the absolute cut-off used for adult, thus defining high-normal blood pressure for SBP and/or DBP 130-139/85-89 mmHg and hypertension for SBP and/or DBP higher than 140/90 mmHg[26]. Very recently, the American Academy of Pediatric recently published new clinical practice guidelines for screening high BP in children and adolescents, proposing significant changes as compared to the European guidelines[27]. In particular, different cut-off points for children above and below 13 years were suggested and new normative tables based on normal-weight children were proposed, beside other important modification in performing screening BP measurements and target organ damage evaluation.

Lifestyle and obesity

Sedentary habits and an unhealthy diet are the main determinants of obesity. In high-income Countries and in several developing countries lifestyle changed in the last few decades determining a reduction in energy expenditure ad an increase in caloric intake due to a higher amount of fat and carbohydrates. Increasing evidence have shown that not only a rich fat diet, but also the quality of fat, can influence the onset of obesity. In particular, a high amount of saturated fatty acids and a lower amount of unsaturated fatty acids contribute to the onset and maintenance of body weight excess[28]. Indeed, in the last century the Western Countries diet increased the total amount of fat and in particular of saturated fatty acids and shifted the ratio between omega-6 PUFA and omega-3 PUFA from 1-

2:1 to 15-20:1, which is thought to increase the susceptibilities to cardiovascular disease and other chronic inflammatory disease[29,30].

In the following paragraphs it will be discussed the biochemical and clinical characteristics of omega-3 and omega- 6 PUFA and pointed out the evidence in children.

Biochemical characteristics and dietary sources of Omega-3 PUFA

Fatty acids are a wide family of compounds with important and manifold biological activities. The difference in chain length and saturation status determines their biochemical characteristics and their biological role. Omega-3 (omega-3) fatty acids are a family of long-chain fatty acids containing more than one carbon-carbon double bond (polyunsaturated fatty acids, PUFA). The main members of this family are α-linolenic acid (ALA, C 18:3), eicosapentaenoic acid (EPA, C 20:5) and docosahexaenoic acid (DHA, C 22:6)[31]. ALA is an essential nutrient for mammals because they lack enzymes where to insert the double bond in omega-3 position; the main dietary sources are plants and plant oils, such as soybean oil, flaxseed oil, canola oil and walnuts[32].

In mammals ALA can be converted to EPA, but only in small percentage, and the conversion of EPA to DHA, if any, is very limited[33]. Accordingly EPA and DHA are considered essential fatty acids, deriving mainly from sea-food, especially high-fat cold-water fish, such as salmon, mackerel, herring and trout[32].

Most of the clinical trials carried out to investigate the potential benefits of omega-3 PUFA in adults and in children have used fish oil as supplements of omega-3 PUFA, however in the last few years the attention was focused also on alternative sources of omega-3 fatty acids, like krill oil, vegetable oils, nuts and algae[34,35].

EPA and DHA enter the food chain trough marine phytoplankton, proceeding through marine mammals and fish, which represent the main dietary source for humans[32].

In natural fish oil EPA and DHA are bond in triacylglycerides (TG). Fish oil capsules are concentrates of marine oils, containing 30-90% of EPA and DHA generally bond in ethyl-ester (EE) or re-esterified TG (rTG)[36,37].

Because of the reported health benefits of EPA and DHA, there is an increasing demand for products rich in marine Omega-3 PUFA and krill oil is an effective sources of these fats.

Krill are small red-coloured crustaceans (*Euphausia superba*) representing the most dominant members of Antarctic zoo-plankton[35]. They are rich in long-chain polyunsaturated fatty acids, 40% of which are EPA and DHA, in form of phospholipids (PL). In addition to Omega-3 PUFA, krill oil contains carotenoid astaxanthin, a potent antioxidant, vitamins A and E, and other fatty acids[38].

The American Food and Drug Administration has classified krill oil as Generally Recognized as Safe and previous clinical and pre-clinical trials have shown that it is safe and well tolerated[38]. Only a few studies have tested the efficacy of krill oil compared with fish oil and the results go in the direction of a possible stronger effect in raising plasma and red blood cell membrane EPA and DHA[35,37,38].

ALA occurs in vegetable food such as nuts, flaxseeds and vegetable oils like canola oil and soy-bean oil, which represent the main sources of Omega-3 in vegetarian diets. However the bioavailability of vegetable Omega-3 FA remains a matter of debate and seems to limit their use as supplements[34]. Indeed, most studies using vegetable sources of Omega-3 (mainly flaxseed oil) report an increase in plasma and red blood cell membranes content of ALA and partially of EPA but not of DHA[39–42]. Moreover findings from short and long-term trials with ALA supplements do not show clear evidence of a protective action in cardiovascular risk and therefore the question whether ALA supplements could be important for cardiovascular health remains unanswered[43].

Observational studies and clinical trials suggest a protective effect of nuts consumption on coronary heart disease and some intermediate biomarkers, such as blood cholesterol and blood pressure[44–46]. The beneficial effect of nuts can be mediated through several mechanisms: nuts are rich in PUFA, with a different content of Omega-6 and Omega-3 in the various types, and in addition they contain fiber, vitamin E, magnesium, potassium and arginine that can contribute to the blood pressure and lipid lowering effect[47].

In the last few years increasing interest was focused on algal DHA-rich oil supplementation: clinical trials reported an increase in plasma and red blood cell DHA content after algal oil supplements, but not of EPA or ALA[34] and, even if in small number, indicate comparable efficacy to fish oil in potential beneficial changes in some markers of cardiovascular risk, in particular on plasma lipid profile [43].

Mechanism of action of omega-3 PUFA

Omega-3 PUFA exert their biological activities through three main classes of mechanisms: some biological effects depend on their incorporation into cell membranes, other effects derive from a direct interaction with ion channels and other cellular components and, finally, EPA and DHA are the parental compounds of bioactive lipid mediators[32]. The exact mechanisms are not yet completely understood, especially it is not clear whether EPA and DHA share the same pathways or not[48].

Omega-3 PUFA can attenuate the response of T-cells and macrophages through cell surfaces receptors, not yet identified, perhaps by changing the composition of membrane microdomains[49]. A direct interaction with some cellular components can mediate some short-term effects such as the antiarrhythmic effect, which depends on a steric interference with ion channels[50], for example the inhibition of the fast, voltage-dependent sodium and L-type calcium currents[49]. Non-esterified Omega-3 PUFA can also directly interact with peroxisome proliferator-activated receptors (PPARs) and others transcription factors, thus modulating gene transcription[48]. Recently a role of GPR120 has been discovered, a member of the family of fatty acid sensing G-protein-coupled receptors (GPCR), mediating the anti-inflammatory and insulin-sensitizing effects of Omega-3 PUFA[51].

Other proteins that can be directly activated by AA and Omega-3 FA, and consequently probably influenced by their ratio, are protein Kinase C, NADPH-oxidase and a two-pore domain K^+ channel[49].

Incorporation of EPA and DHA into cell membranes can modulate the properties of lipid rafts and thus alter the membrane fluidity, affecting hormone receptor binding and the function of membrane-associated proteins[32].

Moreover, EPA and DHA, mostly incorporated into the second position of membranes phospholipids, can be released by phospholipase A2 (PLA2) and converted to a variety of eicosanoids and other lipid mediators through three different metabolic pathways. The first two pathways involve cyclooxygenase (COX) and lipoxygenase (LOX), leading to the formation of prostaglandins, thromboxanes, and leukotrienes; the third branch is catalyzed by cytochrome P 450 (CYP450) leading to the formation of eicosanoids.

Therefore EPA and DHA share the same metabolic pathways of arachidonic acid (AA) and, moreover, it has been shown that they compete with AA for binding with these enzymes, thus inducing profound changes in metabolites biosynthesis that could in part explain the beneficial actions of Omega-3 compared to Omega-6 FA[48]. The competition of EPA and DHA with AA may determine the synthesis of TXA3, almost inactive, instead of TXA2, which has pro-aggregatory properties. Furthermore, starting from Omega-3 FA the metabolism via COX lead to the formation of PGI3 that share the antiaggregatory effect of the AA-derived PGI2. The metabolism of EPA via LOX determine the biosynthesis of LTB5, less potent than the pro-inflammatory AA-derived LTB4.

Moreover, Omega-3 PUFA are also the precursors of novel families of compounds, the so-called resolvins, protectins and maresins, with anti-inflammatory and pro-resolving properties[52]. In particular EPA and DHA, through the complex metabolism involving COX-2 and aspirin-dependent formation of intermediate metabolite, followed by a conversion via LOX, are metabolised to the E-series of resolvins, starting from EPA, and to D-series of resolvins, protectins and maresins from DHA, which counteract the excessive inflammatory while regulating the trafficking of leukocytes and stimulating non-inflammatory phagocytosis of apoptotic neutrophils by macrophages[53].

CYP enzymes accept EPA and DHA as efficient substrates alternative to AA. CYP epoxygenases produce epoxyeicosatrienoic acids (EETs) from AA, epoxyeicosatetraenoic acids (EEQs) from EPA and epoxydocosapentaenoic acids (EDPs) from DHA. CYP hydroxygenases lead to the biosynthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) from AA, 20-hydroxyeicosapentaenoic acid (20-HEPE) from EPA and 22-hydroxydocosahexaenoic acid (22-HDoHe) from DHA[48]. The role of epoxy- and hydroxy-metabolites of AA in cardiovascular function is well known: EETs are mainly involved in antihypertensive and organ-protective mechanisms, in particular they determine vasodilatation in the systemic vascular system, natriuresis in the kidney and act as endothelium-derived hyperpolarizing factors. 20-HETE is involved in both anti- and pro-hypertensive mechanisms, depending on the site of formation and action.

It shares with EETs the natriuretic effect but determines vasoconstriction in the vessels[52].

The evidence shows that LOX, COX and CYP enzymes have the ability to metabolize EPA and DHA instead AA, but the relative efficiencies are not well understood. EPA and DHA are generally considered as poor substrates for LOX and COX, compared with AA, whereas they are efficiently metabolized by CYP enzymes with similar or higher rates, compared with AA[48]. A recent study on 20 healthy volunteers indicate that CYP epoxygenases metabolize EPA with an 8.6-fold higher efficiency and DHA with a 2.2-fold higher efficiency than AA, whereas the effects on leukotriene, prostaglandin E, prostacyclin, and thromboxane formation remained rather weak[54]. The evidence of the clinical significance of EPA/DHA metabolism via CYP450 under *in vivo* conditions is still rare and further investigation is needed.

In animal models, the biological activities of these compounds are partially overlapped to the AA-derived counterparts and partially specific: EEQs and EDPs can be as strong as EETs in vasodilation or even stronger in some vascular beds like cerebral and coronary vessels[55,56]. In an animal model of angiotensin II-dependent hypertension it has been shown a possible role of one EDP isomer as a mediator of the antihypertensive effect of DHA[57]. Some EPA-epoxydes exert anti-inflammatory properties like some EETs regioisomers[58]. Moreover some regioisomers of EEQs and EDPs modify the contractility of neonatal cardiomiocytes, indicating a possible antiarrythmic effect[59].

The suggested hypothesis is that CYP-dependent epoxy-metabolites of EPA and DHA may contribute to the vasodilatory and cardioprotective effects of Omega-3 fatty acids and could also serve as biomarkers of EPA/DHA supplementation.

Finally, the results of a recent randomized controlled trial on patients with peripheral vasculopathy suggest another possible mechanism: after 6 months flaxseed oil supplementation, rich in ALA, plasma eicosanoid profile changed with a decrease in soluble epoxide hydrolase products compared with controls and these subjects exhibited a significant decrease in systolic BP. The authors' conclusion is that flaxseed oil may inhibit soluble epoxide hydrolase thus modifying the lipid mediator concentrations that can contribute to the antihypertensive effect[60].

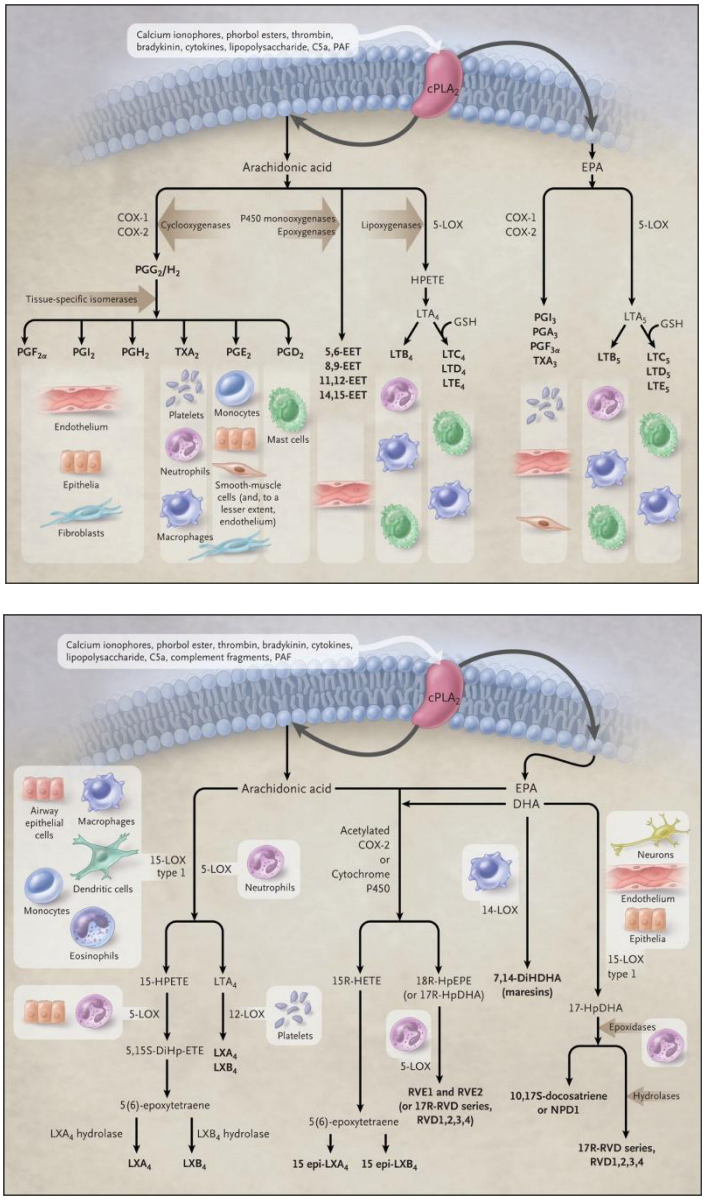


Figure 2. Reproduced with permission from De Caterina R, *NEJM* 2011, Copyright Massachusetts Medical Society [32]

Metabolic pathways involve reactions catalyzed by conversion through cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and lipoxygenases to eicosanoids, which include prostaglandins, thromboxanes, and leukotrienes in different cell types. When released from membrane phospholipids, n–3 fatty acids can give rise to a host of novel eicosanoid derivatives through metabolism catalyzed by several lipoxygenases, modified cyclooxygenases, and the cytochrome P450 system, producing hydroxy- and hydroperoxy-derivatives, as well as lipoxins, maresins, protectins.

COX-2 denotes cyclooxygenase-2, cPLA2 cytosolic phospholipase A2, EET epoxyeicosatrienoic acid, GSH glutathione, HPETE hydroperoxy-eicosatetraenoic acid, LT leukotriene, PAF platelet-activating factor, PG prostaglandin, DHA docosahexaenoic acid, DiHDHA dihydroxy-docosahexaenoic acid, DiHp-ETE dihydroperoxy-eicosatetraenoic acid, EPA eicosapentaenoic acid, HETE hydroxy-eicosatetraenoic acid, HpDHA hydroperoxy-docosahexaenoic acid, HpEPE hydroperoxy-eicosapentaenoic acid, HPETE hydroperoxy-eicosatetraenoic acid, LOX lipoxygenase, NPD1 neuroprotectin D1, and RV resolvin.

Clinical effects of omega-3 PUFA

Omega-3 FA have a wide range of actions as follows: they may act on the vessels by improving the endothelial function and elastic properties of the arteries [61], exerting a favorable effect on the autonomic system and reducing platelet aggregation [62], exerting anti-arrhythmic action by increasing the arrhythmic thresholds [63] and playing an important role in the modulation of the inflammatory response [64]. Finally, they may improve the serum lipid profile [65]. In summary, the rising interest in omega-3 FA is due to their pleiotrophic effects and to the possible eventual protective effect on cardiovascular disease, although the available data are not always consistent. In fact, despite observational studies that have mostly shown a protective effect, the results from randomized controlled trials (RCT) have not always been uniform and successive meta-analyses have questioned the presence of a large omega-3-FA effect in at-risk populations.

For example, a recent large prospective study conducted on healthy older adults investigated the role of omega-3 FA consumed through the typical diet in primary prevention. The results showed that plasma omega-3 FA, considered as single FA (EPA, DHA and DPA) and total omega-3 FA, were associated with lower total mortality, largely dependent on fewer cardiovascular deaths and in particular fewer arrhythmic deaths [66]. Moreover, total mortality was inversely associated with EPA and DHA in a linear fashion, whereas the inverse association with total omega-3 FA was not linear, in accord with previous studies [67]. However, it should be considered that circulating FA are influenced by short-term fluctuations in dietary FA consumption, which probably affects the true relationship between plasma FA and mortality.

Additionally, some large interventional trials supported the beneficial effect of omega-3 FA in cardiovascular disease as follows: in the GISSI-Prevenzione trial, the treatment by omega-3 FA lowered the risks of death, non-fatal myocardial infarction and stroke in a sample of 11,324 patients surviving a recent myocardial infarction [68]. Another trial, investigating the effect of EPA supplementation in primary prevention in 14,981 hypercholesterolemic subjects on statin therapy, indicated a reduction in the incidence of coronary artery disease, particularly in the patients with a high-risk dyslipidemic pattern (high triglyceride level and low HDL-cholesterol level) [69]. Regardless, recent meta-analyses, including a large number of primary studies, have not demonstrated unequivocal results concerning cardiovascular risk [70–72]. In a systematic review and meta-analysis, including 20 studies with a total of 68,680 patients, omega-3 FA supplements were not associated with a lower risk of all-cause mortality, cardiac or sudden death, myocardial infarction or stroke [70]. Another meta-analysis, including 14 randomized placebo-controlled trials involving 20,485 patients with a history of cardiovascular disease, reported insufficient evidence of a beneficial effect of omega-3 FA supplements in secondary cardiovascular prevention [72]. However, it is worth considering that the trials included in the meta-analysis had a short follow-up period (2 years or less) and that the above-mentioned large trials [68,69] with positive findings were excluded from some meta-analyses because they were not placebo-controlled, suggesting a cautious interpretation of the results. The most recent meta-analysis on the same topics arrived at the same

conclusion when considering the association between coronary risk and fatty acids from dietary intake, assessed by questionnaire or using dietary records, or by circulating levels of fatty acids. Comparing the participants in the top third to those in the bottom third, dietary long-chain omega-3 FA and plasma levels of EPA and DHA were associated with a nearly 13% lower coronary risk[73]. When assessing the effect of omega-3 FA supplements in RCT, no significant reduction in coronary risk was found[73].

Uncertainty also remains regarding the relationship between fish or omega-3 FA consumption and cerebrovascular disease. A systematic review and meta-analysis of 26 prospective cohort studies and 12 RCT, which investigated the relation between the risk of cerebrovascular disease and omega-3 FA and fish consumption in primary and secondary prevention, showed the presence of a moderate, inverse association [74]. The analysis of the prospective trials indicated a protective effect of fish intake with regard to the risk of cerebrovascular disease in the general population and showed that an increment of fish intake (at least 2 servings per week) reduced cerebrovascular risk by 4%. Interestingly, the analysis of the different type of fish consumed showed a possible favorable effect of fatty fish, whereas such findings were not significant for white fish. Conversely, the RCT analysis of omega-3 FA supplementation did not indicate any significant correlation with cerebrovascular risk [74].

The evidence concerning the beneficial effect of EPA/DHA on BP appears clear as follows: many interventional studies with fish oil have suggested a beneficial effect of omega-3 supplements on BP control, particularly in hypertensive subjects, with a possible threshold effect. The first meta-analysis to show a positive antihypertensive action of omega-3 FA in hypertensive patients, compared to normotensive subjects, was reported by Morris et al. in 1993 [75].

These results were consistent with a subsequent meta-analysis of 36 studies evaluating a total sample of 2,114 subjects receiving high doses of fish oil (mean dose 4.1 g/day) as follows: fish oil significantly reduced BP (SBP - 2.1 mmHg and DBP - 1.6 mmHg), with a stronger effect in hypertensive and older (> 45 years) subjects. Interestingly, no dose-response relationship was observed and the effect of low doses (< 500 g/day) of omega-3 supplements remains unclear [76]. They observed a dose-response effect when the studies were grouped by Omega-3 FA dose; however, it should be emphasized that the omega-3 dose was relatively high in the group receiving the lowest dose (up to 3 g/day).

The most recent meta-analysis examined 70 randomized controlled trials with EPA + DHA supplements in hypertensive and normotensive subjects [77]. The mean dose of EPA and DHA was 3.8 g/day, deriving mostly from fish oil and also from EPA- and DHA-fortified foods, seafood and algal oil. Omega-3 supplements provided a reduction in systolic BP (SBP) of 1.52 mmHg and diastolic BP (DBP) of 0.99 mmHg in the meta-analysis of all studies with hypertensive and normotensive subjects, compared with placebo (mostly olive oil and other vegetable oils). The analysis of untreated hypertensive subjects showed the strongest effect for EPA+DHA in lowering BP, compared to normotensive subjects [77].

Prospective cohort studies have also examined the impact of the omega-3 FA dietary content on the development of hypertension in normotensive subjects, with some finding an inverse association [78,79] and others no association[80,81]. A meta-analysis, including 8 of these studies with approximately 56,000 subjects followed up for 3 to 20 years, showed that the subjects with the highest dietary consumption of omega-3 FA, as determined by plasma or erythrocyte fatty acid content, had a lower risk of developing hypertension compared to subjects with the lowest intake[82]. Interestingly, this finding supports the hypothesis of a stronger protective effect for DHA in primary prevention.

A recent study on 312 subjects, evaluating the impact on BP of lower doses of EPA + DHA (0.7 and 1.8 g/day, respectively), which are more easily achievable through dietary modification, showed that only patients with isolated systolic hypertension exhibited a significant reduction in SBP (- 5 mmHg)[83].

Current guidelines by the American Heart Association Nutrition Committee suggest the consumption of at least 2 servings of fish per week for primary cardiovascular prevention and Omega-3 supplements for secondary prevention [84,85]. Regarding BP, the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC) guidelines published in 2013 recommend that patients with hypertension eat fish at least twice a week[86], without specifying the type of fish, whereas the 7th and 8th Joint National Project reports mention neither fish nor omega-3 FA[87,88].

In addition to the actions of EPA and DHA, recent studies found a BP-lowering effect of flaxseed oil, which is rich in ALA as follows: in a double-blind, placebo-controlled RCT (FlexPAD trial) in 110 patients with peripheral artery disease (PAD), the treated group (30 g/day of flaxseed) showed an increase in plasma ALA, which was inversely associated with BP, and they exhibited a decrease in SBP and DBP after 6 months that was significant in hypertensive patients [89]. A subsequent study on the same population in the FlexPAD trial moreover displayed a reduction in central SBP and DBP after flaxseed supplementation[90].

The possible stronger effect of dietary fish on BP, compared to EPA/DHA supplementation, raises some unanswered questions. First, it warrants consideration that the biological role of single nutrients probably cannot be separated from the wide range of other substances contained in a food. For example, fish is rich in vitamins [91], amino acids [92] and some trace elements [93] that may also exert favorable vascular effects. Second, the intake of certain foods should be assessed in the context of the whole diet, considering the balance between the different nutrients that may exert a variety of actions and interactions.

Vascular effects of omega-3 FA

A clinically relevant action of omega-3 FA is the BP-lowering effect, which is believed to derive from reduced systemic vascular resistance and improved endothelial function, thus interfering with the atherosclerotic process [94]. The modulation of vascular functions remains, in our opinion, one of the key aspects of cardiovascular protection by omega-3 FA.

Studies on animal models suggest that EPA, but probably not DHA and DPA, may induce Ca++-independent activation and translocation of endothelial nitric oxide synthase (eNOS) with consequent endothelium-dependent vasodilation [95]. In vivo, EPA induces an increase in eNOS phosphorylation via the up-regulation of uncoupling protein-2 (UCP-2) and activation of AMP-activated protein kinase [96]. In the past few decades, dozens of studies in humans were conducted with divergent results concerning the effect of omega-3 FA on endothelium-dependent vasodilation, whereas no effect was consistently observed regarding omega-3 FA supplements on endothelium-independent dilation. Two meta-analysis, including 16 clinical trials, show improvement in endothelial function after omega-3 FA supplementation [97,98]. However, this result warrants a careful evaluation as follows: first, in the meta-analysis conducted by Wang and colleagues, in addition to trials with EPA + DHA, studies with ALA supplements were also included, whereas Xin and colleagues considered only EPA + DHA supplementation for the analysis. Moreover, the sample size in most of the primary studies was low, and the significance of the results appeared mainly dependent on the contribution of the low-quality studies[99,100]. Regardless, both meta-analyses included a high-quality randomized double-blind trial in 312 healthy non-smoking subjects with increasing doses of EPA + DHA (0.45, 0.9 and 1.8 g/day)[101]. Compliance with the intervention was verified by a significant increase in the EPA and DHA content of red blood cell membranes; however, endothelial function, as measured by Flow Mediated Dilation (FMD), was not related to EPA+DHA intake[101].

Thus, further studies and large-scale RCT remain required to define the role of omega-3 FA on endothelial function. Moreover, the evidence concerning the effect of EPA and/or DHA on arterial stiffness suggests a possible protective effect, though the clinical significance remains to be clarified. The largest observational study was conducted in a subcohort of the Framingham study, including 3,055 subjects as follows: a moderate association between red blood cell omega-3 FA content and several measures of arterial stiffness was observed, in particular, the carotid-femoral pulse wave velocity, which is considered the gold standard index of aortic stiffness. However, after multivariable adjustment, only a modest correlation between higher omega-3 content in red blood cell membranes and lower aortic stiffness remained [102]. A recent small interventional study on 29 subjects receiving EPA + DHA supplements (2 g/day) reported a significant improvement not only in flow-mediated dilation but also in pulse wave velocity, a measure of aortic stiffness [103]. Despite the scarce and mostly underpowered RCT in humans, a recent meta-analysis appears to confirm the association between omega-3 FA supplements and arterial stiffness and arterial compliance; interestingly, the results were not affected by BP changes, indicating a possible BP-independent effect of omega-3 FA on arterial function [104].

Moreover, epidemiological and experimental studies suggest that EPA and DHA are associated with less vascular calcification, probably due to their preventive effect on tissue remodeling [105–107].

A population-based observational study on 1,570 subjects in the Netherlands investigated the correlation between fish and EPA + DHA intake, assessed using a 170-item semiquantitative food-frequency questionnaire and coronary calcification measures determined by CT scanning according to Agatston's method. The investigators found that subjects with a higher fish intake had a significantly lower prevalence of mild to moderate coronary calcification compared to subjects who did not consume fish, whereas EPA + DHA intake showed no significant association [108]. No interventional trials on humans are available to date to determine the effect of EPA and DHA on vascular calcifications.

Clinical effects of omega-3 in children

The incidence of obesity and type 2 diabetes reported in children has increased in the last few decades and it has been shown that obesity plays a pivotal role in the development of insulin resistance, which is related to hyperinsulinemia, hypertension, hyperlipidemia, type 2 diabetes and increased risk of atherosclerotic disease. Moreover considerable evidence shows that overweight in childhood and adolescence is associated with insulin resistance, dyslipidemia and high blood pressure in young adults[109].

In the last few years, there has been emerging interest also for the possible beneficial effect of Omega-3 in childhood with respect to cardiovascular risk factors.

Some observational studies and a limited number of interventional studies indicate a positive effect of Omega-3 supplements on **blood pressure** control, even if the data currently available are small and not unequivocal (see Table 1). A recent cross-sectional study on seventy-three 8-11 year-old Danish children shows a positive association of mean arterial pressure with whole-blood DHA only in boys and this correlation remains also after adjustment for energy intake, body-fat percentage and physical activity. OMEGA-3 FA were measured in whole-blood and were found to be associated with fish intake, recorded for 7 days by a Web-based dietary assessment specifically for children[110].These findings were in agreement with a previous randomized trial of the same group, showing a decrease in systolic blood pressure in healthy 12-mo children (n=83), after 3 months of fish oil supplements (mean estimated EPA and DHA assumption: 924 mg/day). Infants were randomly assigned to fish oil supplements or not and to two different milk types and the effect of fish oil supplementation was adjusted for the effects of milk intervention. The fish oil supplements were also inversely associated with plasma triglycerides and directly with total cholesterol and LDL cholesterol[111]. A positive effect on systolic and diastolic blood pressure was found also in adolescent boys aged 13-15 after a 16-week fish oil supplementation (1.5g EPA and DHA) compared with the control group receiving vegetable oil[112]. In contrast, a cross-sectional study in a hundred and nine 17 year-old

adolescents found a poorer metabolic profile, included higher systolic blood pressure levels, associated with a higher DHA content in red blood cell membranes, which remained also after adjustment for physical activity and dietary factors [113].

Data from the National Health and Nutrition Examination Survey indicate that a higher dietary intake of EPA and DHA, recorded by two 24-hours dietary recall, in 354 children, aged 8-15 years, born with reduced birth weight, are associated with lower systolic blood pressure and pulse pressure[114]. These findings were consistent with a previous exploratory analysis, which showed a significant inverse association of serum Omega-3 PUFA with systolic blood pressure in young adults, aged 24-39 years, born with impaired foetal growth[115]. These data suggest that Omega-3 PUFA could play a role in blood pressure control in subjects with low birth weight, which is a known factor independently associated with an increased risk of cardiovascular events in adulthood[116].

A cross-sectional study performed on 814 Australian adolescents (13-15 year-old) suggests a possible role of gender in modulating the relationship between Omega-3 FA and blood pressure: systolic and diastolic blood pressure were inversely associated with EPA and DHA intake, assessed with a 3-day diet record, in boys but not in girls[117].

Furthermore, an association between blood pressure and the Omega-3 content was found in boys but not in girls also in the above mentioned cross-sectional study conducted by Damsgaard[110] and in a trial in breast-fed infants of mother receiving Omega-3 supplements, but the latter found an unfavourable relation of fish oil supplements with blood pressure control[118].

Some studies have investigated the long term effect of Omega-3 FA supplements administered in early infanthood through lactation or to the mothers during pregnancy but the results are not unequivocal. The interest stemmed from evidence that breast feeding, rich in EPA and DHA, in contrast to the first formula milk, is associated with lower blood pressure in childhood and adulthood[119,120].

In an interventional study, children who received human milk with an Omega-3 FA content above the median had a nearly 5 mmHg lower systolic and 2.5 mmHg diastolic blood pressure at the age of 12 years compared with never breast-fed children, but Omega-3 FA content below the median in the milk was not associated with blood pressure levels at 12 years[121]. In contrast, in a previous trial boys of mother receiving fish oil supplements during the first 4 months of lactation showed a higher diastolic blood pressure at the age of 7 years compared with the olive oil control group[118].

In another study investigating the long-term effects of Omega-3 supplements, the supplements of Omega-3 FA from infancy to 5 years (canola oil and tuna oil), with a contemporary reduction of omega-6 FA assumption in order to provide an Omega-3 to Omega-6 ratio of 1:5, do not affect blood pressure and vascular structure at the age of 8 years, even if the concentration of Omega-3 FA in the plasma were higher in the intervention group compared with controls[122].

Omega-3 FA supplements are effective in lowering triglyceride levels in adults, therefore in international guidelines their consumption is suggested for patients who need to lower triglycerides. No effects on the other plasma lipid levels such as cholesterol are evident in adults. Little is known about the **lipid lowering action** of EPA and DHA in children and adolescents, only a small number of clinical trials are available and the results are not always encouraging. In two recent interventional studies in children and adolescents with hypertriglyceridemia (n= 111 and n=25 respectively), aged 8-18 and 9-19 years old respectively, 3-6 months fish oil supplements, at the doses of 500-1000 and 3360 mg per day, did not lower plasma triglycerides compared with control group[123,124].

In contrast a positive effect in lowering plasma triglycerides was found in 103 obese children and adolescents after 12 weeks of 1.8 g/day of Omega-3 supplements, compared with the control group receiving metformin, and the other plasma lipids were not affected by the treatment[123,124].

The evidence of an action of Omega-3 FA on cholesterol is scanty and not conclusive: Damsgaard found a positive correlation between EPA levels and HDL levels in 8-11 years-old children[110]. However in a previous study fish oil supplements were associated to higher total and LDL cholesterol in 9-12 months infant[111]. In an observational study no association was found between fish consumption, assessed by a 7 day pre-coded food diary, and serum lipid profile in hundred-and-nine adolescents[113].

A large amount of data, from epidemiological studies, indicate a protective effect of Omega-3 FA on glucose metabolism and **insulin sensibility** in adults[125,126]. Accordingly, the results of many clinical trials support the positive effect of Omega-3 on glycemic control, however some showed negative results[127,128]. The evidence from observational studies and clinical trials in children is limited but supports a beneficial effect of Omega-3 also during childhood (see Table 2).

A cross-sectional study on 5-12 years-old children showed a moderate but significant correlation between fasting insulin levels and HOMA-IR and the Omega-3 Index, which is the percentage of EPA and DHA contained in red blood cell membranes, as an index of chronic Omega-3 intake[129]. Interestingly, in this population, obese children had altered erythrocyte fatty acid composition unrelated to reported dietary intake and showed lower levels of Omega-3 Index compared with non-obese children[129].

Moreover, lower plasma and erythrocyte membrane levels of AA and DHA were found in 40 diabetic children compared with non-diabetic controls[130]. However in a longitudinal study conducted on 167 children at increased genetic risk for type 1 diabetes for the development of persistent islet autoimmunity, Omega-3 and Omega-6 FA levels in red blood cell membranes were not associated with the development of type I diabetes[131]. In a clinical trial on 76 overweight and insulin resistant children the supplementation with 900 mg per day of Omega-3 FA for 1 month decreased fasting insulin and HOMA-IR, also after adjustment for pubertal status and weight loss[132]. Accordingly, a recent trial showed a

reduction of glucose and insulin levels while reducing HOMA-IR after 12 weeks of 1.8 g of Omega-3 supplementation in 201 obese and insulin resistant children, also after adjustment for sex, age and change in BMI[124].

There is also increasing interest on the use of Omega-3 supplements in the treatment of **non-alcoholic fatty liver disease** (NAFLD), which is pathogenically linked to insulin resistance and metabolic syndrome. According to the evidence of a positive effect of EPA and DHA supplements on liver steatosis in adults, also the few clinical trials available in children support the hypothesis that DHA can decrease liver fat content in children with NAFLD[133,134].

Animal models show positive results encouraging the use of Omega-3 FA to prevent diet-induced **obesity**[135] and some trials in overweight adults and in obese diabetic women have also reported a beneficial effect in fat mass reduction[136,137]. Nowadays, there is no clear evidence in children and the attention is mainly focused on the possible programming effect of Omega-3 fatty acids in breast milk on later infant and young children body composition. The results from the available studies are divergent: some studies show an inverse correlation between maternal Omega-3 FA intake and Omega-3 FA content in formula milk and later body composition[138–140], others show a direct correlation[141–143], finally in some studies no significant correlation was found[118,144–146]. A recent study on 201 obese children and adolescents indicates a beneficial effect of Omega-3 supplements (1.8 g EPA and DHA for 12 weeks), without other lifestyle interventions, on weight reduction[124]. In contrast Nobili reported no effect on BMI after 6 months DHA supplements in children compared with placebo, but the doses of DHA were low (250-500 mg/day)[134].

Table 1. Studies about the effect of Omega-3 PUFA on blood pressure.

Author, year	Study design and aim	No. subjects	Source and dose	Time period	BP outcome
Damsgaard CT, 2013[110]	Cross-sectional study on 8-11 y-o children. To investigate the relationship between whole-blood EPA and DHA and Metabolic Syndrome features	73 (F=44, M=29)	Fish intake assessed by a specific pediatric dietary assessment; amount consumed estimated by portion size among 4 different images	---	Positive association of mean arterial pressure with DHA only in boys after adjustment for energy intake, body-fat percentage and physical activity
Damsgaard CT, 2006[111]	RCT: 9-12 mo infants randomly assigned to fish oil or no supplements and to cow's milk or infant formula. To investigate the effect of fish oil on BP and lipid profile in infants	83 (F=42; M=41)	Fish oil (mean estimated EPA and DHA assumption: 924 mg/day)	3 months	Lower SBP (-6.3 mmHg) in infants administred fish oil, also after adjustment for milk intervention
Pedersen MH, 2010[112]	RCT on 13-15 y-o boys with a control group receiving vegetable oil. To investigate the effects of fish oil on cardiovascular risk factors	78 (F=0; M=78)	Fish oil (1.5 g/day long-chain Omega-3 FA)	16 weeks	Lower SBP and DBP (-3.8 and -2.6 mmHg respectively) in fish oil group
Lauritzen L, 2012[113]	Cross-sectional study on 17 y-o adolescents. To investigate the association between fish intake and Metabolic Syndrome features	109 (M=44; F=109)	Fish intake assessed by 7 days food record with pre-coded response categories; intake registered in household measures and portion size based on images	---	Higher DHA status correlated with higher SBP, after adjustment for sex, body fat percentage, dietary factors and physical activity
Skilton RM, 2013[114]	Cross-sectional study on 8-15 y-o children born with low birth weight. To investigate the relation between Omega-3 FA and BP in children with relative hypertension related to reduced birth weight	354 (F=174;M=180)	Fish intake assessed by two 24-h dietary recall, the second after 3-10 days	---	Children in the highest tertile of dietary EPA and DHA intake had significantly lower SBP (−4.9 mm Hg and pulse pressure (−7.7 mm Hg) than children in the lowest tertile

Table 1 – continued

Author, year	Study design and aim	No. subjects	Source and dose	Time period	BP outcome
O'Sullivan TA, 2012[117]	Cross-sectional study on 13-15 y-o adolescents. To investigate the relation between Omega-3 FA and BP	814 (F=395;M=419)	Three-day diet record measured in household units	---	Inverse association between Omega-3 FA and SBP, DBP and mean arterial pressure in boys
Ayer JG, 2009[122]	RCT on children in the first 5 years, randomly assigned to fish oil and reduction of Omega-6 FA or control (sunola oil). To investigate BP and arterial structure and function in 8 y-o children who received Omega-3 FA supplements in the first 5 years of life	410 (F=203;M=207)	Canola oil and tuna oil, doses depending on age; every tuna oil capsule had 6% EPA and 27% DHA.	Follow-up at the age of 8 years	No significant differences in BP between intervention group and control group
Van Rossem L, 2012[121]	Observational cohort-study in breast-fed children with a never-breast-fed children control group. To investigate the association between fatty acid composition of infant milk feeding and blood pressure at the age of 12 yeasr.	314 (F=161;M=153)	Assessment of fatty acid composition of human milk. Infant formula (control) did not contain Omega-3 FA	Follow-up at the age of 12 years	Children who received human milk with an n-3 long-chain polyunsaturated fatty acids content above the median had a 4.79-mm Hg lower systolic and a 2.47-mm Hg lower diastolic blood pressure at age 12 years than control
Asserhøj M, 2009[118]	DB-RCT on mother receiving fish oil or olive oil (control). To investigate whether fish oil supplements during lactation affect BP and body composition of children	175 (F=175; M=0)	Fish oil (0.6 g/d EPA and 0.8 g/d DHA)	Fish oil supplements in the first 4 months of lactation. Children follow-up at 2.5 y and 7 y.	FO boys had 6 mm Hg higher DBP and mean arterial BP than controls, but girls

F, female; M, male; y-o, years old; Omega-3 FA, omega-3 fatty acid; RCT, randomized controlled trial; DB-RCT, double blind- randomized controlled trial; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; EPA, epoxyeicosatrienoic acid; DHA, docosahexaenoic acid.

Table 2. Studies about the effect of Omega-3 PUFA on insulin sensitivity.

Author, year	Study design and aim	No. subjects	Source and dose	Time period	Insulin resistence outcome
Burrows T, 2011[129]	Cross-sectional study on 5-12 y-o children. To investigate the relationship between Omega-3 Index, weight and insulin resistance	48 (F=28; M=20)	Fat intake assessed by a 135-item semi-quantitative food frequency questionnaire	---	A moderate correlation found between Omega-3 Index and fasting insulin level and HOMA-IR.
Decsi T, 2002[130]	Observational study on 8-16 y-o diabetic children. To compare plasma and red blood cell membranes fatty acids in diabetic children with non diabetic controls	80 (F=50; M=30)	Plasma and red blood cell membranes determination of AA, EPA, DHA and ALA	---	Lower levels of AA and DHA in diabetic children compared with controls
Miller MR, 2011[131]	Longitudinal study on 0-8 y-o children with islet autoimmunity. To investigate the correlation between Omega-3 FA intake and erythrocyte membrane Omega-3 fatty acid levels and type 1 diabetes	167 (F=82; M=85)	Dietary intake assessed by a 111-item semi-quantitative food frequency questionnaire	---	No significant association between Omega-3 FA and Omega-6 FA erythrocyte membranes levels and the onset of type 1 diabetes
Lòpez-Alarcòn M, 2011[132]	RCT on 9-18 y-o overweight and insulin resistant children. To investigate the effect of Omega-3 FA supplements on body weight and insulin resistance, compared to placebo group	76 (M; F not specified)	900 mg of Omega-3 FA (360 mg DHA + 540 mg EPA)	1 month	Supplementation with Omega-3 FA decreased HOMA-IR by 15% after adjusting for puberty, treatment adherence, changes in adipokines, and weight loss
Juàrez-Lòpez C, 2013[124]	Open-label study on 10-12 y-o obese and insulin resistant children assigned to Omega-3 FA or Metformin (control). To investigate the effect of Omega-3 FA on HOMA-IR and BMI	201 (F=106 ; M=95)	600 mg of Omega-3 FA (360 mg of EPA and 240 mg of DHA)	12 weeks	Reduction of glucose and insulin levels while reducing HOMA-IR in Omega-3 FA group compared to controls, also after adjustment for weight reduction, sex and age.

F, female; M, male; y-o, years old; Omega-3 FA, omega-3 fatty acid; RCT, randomized controlled trial; DB-RCT, double blind- randomized controlled trial; HOMA-IR, homeostasis model assessment-estimated insulin resistance index; EPA, epoxyeicosatrienoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; BMI, Body Mass Index.

Adverse effects of omega-3 PUFA

Omega-3 supplement are generally considered safe and, where reported, the tolerability of Omega-3 FA in clinical trials, in children as well in adults, was good with no major adverse reactions. An adverse effect of Omega-3 FA supplements is the increased risk of bleeding, due to the potential antithrombotic effect, however the evidence available does not support a clinical relevant increased bleeding, even when Omega-3 FA supplements were concomitant with antiplatelet or anticoagulant therapy[147]. Another potential safety concern is the presence of contaminants, especially mercury and dioxins, in fish and fish oil, which has direct implications for dietary recommendation, in particular for some population such as pregnant women and infants[148].

Conclusion and perspectives

In summary, the clinical effect of Omega-3 FA on cardiovascular risk factors in children is not unequivocal. Actual evidence supports a beneficial effect of Omega-3 FA supplements on insulin sensitivity and a possible positive effect on blood pressure control whereas they are not yet conclusive concerning the effect on plasma lipids and body composition. Anyhow, there were few clinical trials in children and most of the evidence comes from epidemiological studies, sometimes with limited sample size. Indeed, doses of supplements varies largely as well as the duration of the interventions, and it is not easy to detect a dose-response effect among different studies.

In humans, different CYP isoforms are responsible for Omega-3 or Omega-6 metabolism, whereas other enzymes, such as the soluble epoxide hydrolase (EPHX2), convert these metabolites to mostly inactive compounds. Polymorphisms in these genes have been tested to evaluate their effects especially in blood pressure homeostasis with some encouraging results[149–157] and it is possible that also preferential dietary intake of Omega-3 vs Omega-6 could influence their effect. Thus, a suggestive hypothesis is that the beneficial effect of Omega-3 FA is not only dependent on their intake and content but also on the complex interaction between different nutrients and polymorphisms in genes involved in Omega-3 FA metabolism[158–166]. These complex interaction has seldom been explored in children and adolescents [162]. Further studies are needed to investigate all these points in order to find a better collocation of Omega-3 FA on the available armamentarium for preventive, possibly individualized, medicine.

Omega–6 PUFA: biochemical characteristics and mechanisms of action

Omega–6 PUFA are a family of polyunsaturated fatty acids that include, as main compounds, linolenic acid (LA) and arachidonic acid (AA). LA is an essential FA, whose main dietary sources are corn and sunflowers oil, nuts and nut oils and poultry[167]. AA is not strictly essential, indeed it can be metabolized in human from LA through desaturation and elongation steps catalyzed by Delta-6 and Delta-5 desaturase[168].

Like omega–3 PUFA, omega-6 PUFA are incorporated into cell membranes, interact with membrane structure, influence intracellular signaling, like the activation of NADPH oxidase, protein kinase C and a two-pore domain K+

channel[169,170]. Moreover, they are further metabolized by LOX, COX and CYP450. AA is normally found incorporated into cell membrane phospholipids and is released by phospholipase A2 and therefore becomes available for the convertion to active lipid mediators. In particular COX converts AA to the "2-series" prostaglandins (PG) (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2) by specific synthase and they act as autocrine or paracrine mediators through a G-protein-coupled receptor (GPCR)[49]. TXA2 has pro-aggregatory effect, whereas PGI2 is anti-aggregatory and vasodilating[171].

LOX are a group of enzymes, classified on the basis of site of AA oxidation; the prominent animal isoforms are 5-, 8-, 12-, and 15-LOX. Through this metabolic pathway AA is converted to the "4-series" of leukotriens (LTB_4, LTC_4, LTD_4, LTE_4, LTF_4) mainly in inflammatory cells and their action is also mediated by (GPCRs). In addition to GPCRs, PG and LT exert their effects also through the activation of peroxisomal proliferator-activated receptors (PPARs)[172,173]. The "4-series" LT is recognized as a proinflammatory cascade, i.e. LTB_4 induces neutrophils chemotaxis and adhesion to endothelium and LTD_4 induces eosinophil chemotaxis[174–176]. PGI2 and PGE2 are also involved in the inflammatory process and evoke hyperalgesia at peripheral and central nervous sites[177,178]. (Figure 3)

AA can be metabolized by the cytochrome P 450 (CYP450) enzymes leading to the formation of EETs via CYP-epoxygenase and to 20-HETE via CYP-hydroxygenase in many tissues, like endothelium, kidney, lung and heart, with possible tissue-specific effects[179–181]. For a widely explanation of CYP450 metabolites of AA see below.

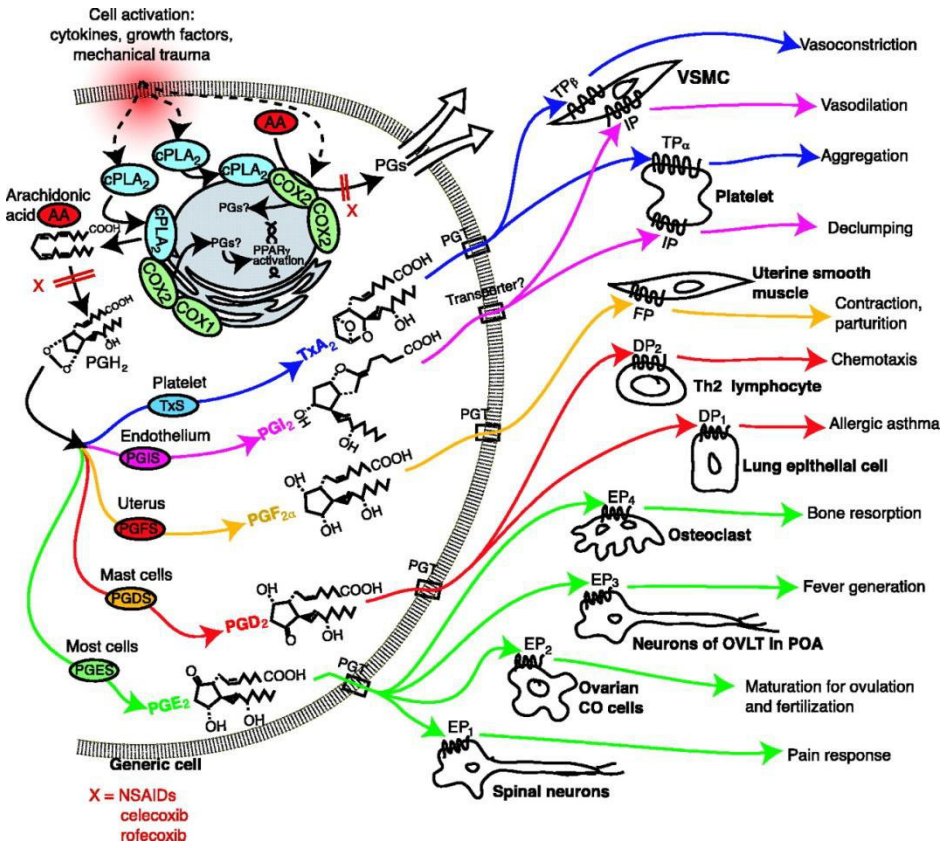


Figure 3. Reproduced with permission from Funk CD, *Science* 2001[172]

Prostaglandin synthesis and actions. A generic cell when activated by mechanical trauma, cytokines, growth factors, or various inflammatory stimuli triggers signaling, including type IV cytosolic phospholipase (cPLA 2) translocation to ER and nuclear membranes, arachidonic acid release from membrane lipids and metabolism by COX-1 or COX-2 to the intermedi-ate PGH 2. Other PLA 2 subtypes could be involved in arachidonic acid release for eicosanoid synthesis but are not shown here. De novo COX-2 enzyme synthesis can be induced by a host of factors (top) to reinforce prostaglandin (PG) formation. In a cell-type restricted fashion, a heterogeneous family of PGH 2 metabolizing enzymes can form PGE 2, PGD 2, PGF 2a, PGI2 (prostacyclin) and TxA2 (thromboxane). These prostaglandins may undergo facilitated transport from the cell through a known prostaglandin transporter (PGT) or other carrier to exert autocrine or paracrine actions on a family of prostaglandin receptors EP 1, EP 2, EP 3, EP 4, DP 1, DP 2 , FP, IP, TP A , and TP B on the cell types indicated. Only a few of the many diverse activities of prostaglandins are shown here. Prostaglandins could potentially enter the nucleus and activate nuclear hormone receptors such as PPAR-g. PGES, PGE synthase; PGDS, PGD synthase; PGFS, PGF synthase; PGIS, prostacyclin synthase; TxS, thromboxane synthase. VSMC is vascular smooth muscle cell. OVLT in POA is the organum vasculosa lamina terminalis at the midline of the preoptic area. CO cells are cells of the cumulus oophorus. X marks the site of inhibition by NSAIDs (aspirin, ibuprofen, indomethacin) and the coxibs celecoxib and rofecoxib

Clinical effects of omega-6 PUFA

In the last few decades a high omega-6/omega-3 PUFA ratio has been associated to an increased CV risk[182], raising the warning to reduce the dietary assumption of omega-6 PUFA, which is recommended to be under 10% of total energy intake according to the current guidelines[183,184]. Anyhow the effects of omega-6 PUFA on the CV system is not to date clearly understood. Principal concerns regard the possible increase in 2-series PG and 4-series LT with a higher intake of omega-6, thus determining an increase in proinflammatory and proaggregatory status, which may worsen the atherosclerotic process. Moreover, previous suggested that there is no correlation between omega-6 FA and the risk of hypertension[80] and that the re-placement of SFA with omega-6 FA is not correlated to vascular and endothelial function[185]. Even arachidonic acid (AA) content was higher in adipose tissue of metabolically unhealthy obese adults as compared to metabolically healthy control[186].

On the other hand, other studies have suggested that omega-6 PUFA might reduce coronary heart disease: i.e. a large study performed in 11 American and European cohorts showed that PUFA were inversely associated with the risk of coronary events, when replacing 5% total energy intake from saturated FA (SFA) with PUFA; whereas the replacement with carbohydrates showed a modest direct association[187]. A longitudinal study showed an inverse relation between an increase in serum omega-6 FA and the prevalence of MetS in a Finnish population[188] and two large cohort studies have seen that dietary intake of omega-6 was inversely associated with total mortality[189]. Some studies indicate that, within omega-6 PUFA, especially linoleic acid (LA) may exert protective actions with respect to body fat distribution, insulin resistance[190], total mortality[189] and blood pressure control[191]. AA, measured in red blood cell membranes, can contribute to glucose homeostasis[192,193], some studies suggesting a beneficial effect only in subject with low-normal insulin sensitivity but not in highly insulin-sensitive individuals[194].

In the current decade three meta-analysis were conducted in order to summarize the role of omega-6 PUFA on cardiovascular disease. A systematic review and meta-analysis of RCTs on 13,614 subjects quantified the effect of replacement of SFA with PUFA on coronary heart disease, showing a 10% decreased risk of coronary heart disease for each 5% energy of increased PUFA in place of SFA, with a greater benefits from studies of longer duration at the meta-regression analysis[195]. Anyhow a meta-analysis of observational, prospective studies and RCTs did not show a significant impact of omega-6 PUFA on coronary disease[73].

Furthermore, a Cochrane review on 660 participants found scarse evidence of an effect of increased or decreased omega-6 PUFA intake on blood pressure and plasma lipids. However this data should be carefully taken into account because of the low number of included studies (four trials)[196].

Clinical effect of omega-6 PUFA in children

Little is known about the role of omega-6 PUFA on cardiovascular system in childhood and only a limited number of studies were conducted in children and adolescents.

In an observational study 67 obese children had lower plasma omega-6 PUFA than the normal weighted controls[197]. In another work also plasma AA was lower in diabetic children with respect to healthy controls, whereas in the same population LA was higher than in control[130]. On the other hand, a recent study on 3-6 months infant with islet immunity showed that higher serum AA/DHA ratio and omega-6/omega-3 ratio was associated with an increased risk of type 1 diabetes[198]. Consistent with these results, a study in healthy children indicates that AA, measured in adipose tissue and in skeletal muscle cells, was directly correlated to fasting insulin and HOMA-IR[199]. Anyhow the scanty number of studies, the lack of RCTs, the differences in the examined populations and in the utilized methods suggest a cautious interpretation of these findings and the need for further investigations.

Omega-6 and omega-3 fatty acids and Cytochrome p450-derived eicosanoids in cardiovascular diseases

In the following paragraphs it will be discussed the so-called third branch of metabolism of PUFA and the interaction between omega-6 and omega-3 PUFA pathways.

CYP450-derived metabolites of AA

The metabolites of AA generated through the three pathways and their clinical and biological effects are better understood compared to those derived from EPA and DHA. In this paragraph, we briefly review the evidence concerning the AA-derived metabolites via CYP450, which have well known hemodynamic effects and, notably, may interfere with the formation of the eicosanoids of EPA and DHA.

COX and LOX metabolize AA leading to the production of prostaglandins, prostacyclin, thromboxane and leukotrienes, which are involved in the modulation of pulmonary and renal function, and in vascular tone and inflammation [200]. Less attention was directed to the third metabolic pathway, the cascade leading to the formation of CYP-derived AA metabolites. In fact, CYP epoxygenase catalyzes the formation of epoxyeicosatrienoic acids (EETs), whereas CYP hydroxygenase leads to the biosynthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) [52]. EETs are generally involved in protective mechanisms and exert an antihypertensive effect as follows: in most vascular beds, they act mainly as endothelium-derived hyperpolarizing factor (EDHF) and may also activate eNOS, leading to a vasodilatory effect [201]. Moreover, they exert a Na+-excreting action[200]. At the renal level, 20-HETE shares the Na+-excreting effect with EETs; conversely, in the vascular system, 20-HETE constricts renal, cerebral, mesenteric and skeletal muscle arterioles [200]. Consequently, 20-HETE acts in opposite directions, eliciting both pro- and anti-hypertensive effects.

Furthermore, EETs mediate anti-inflammatory actions [202] and their less potent metabolites dihydroxyeicosatrienoic acids (DHETs), produced by the soluble Epoxide-Hydrolase (sEH), have antithrombotic effects and may be involved in the inflammatory process, with some reports suggesting them to be pro-inflammatory [203] (Figure 4). Whereas a number of studies in animal models recognized a role of 20-HETE and/or EETs in the development of hypertension [204,205], to date a small number of human studies are available . In renovascular hypertension in particular, some clues were unveiled regarding the involvement of CYP-derived metabolites of AA, notably 20-HETE. [206]. Two studies indicated the involvement of CYP-derived metabolites of AA in the regulation of the maternal circulation during pregnancy and a possible contribution to pre-eclampsia, although their pathophysiological role is not defined to date[207,208].

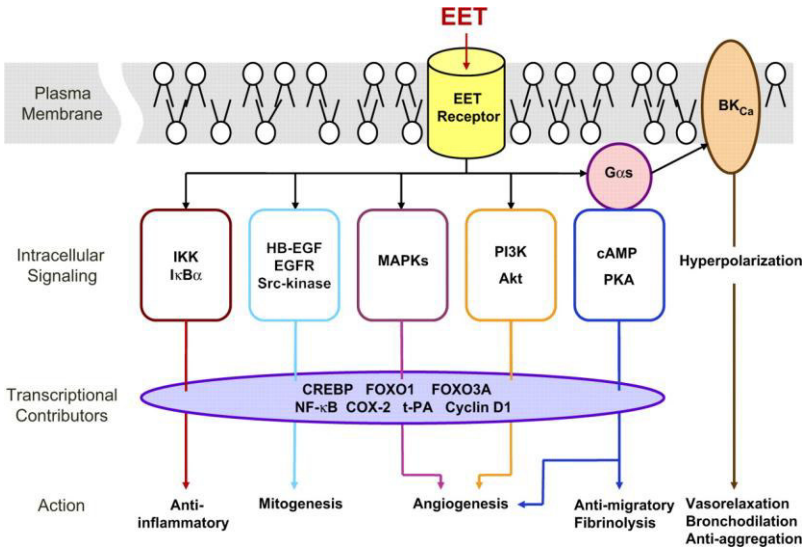


Figure 4: Reproduced with permission from Spector AA et al. *Am J Physiol Cell Physiol* 2007

Membrane receptor mechanism of EET action. The key element in this mechanism is EET binding to a putative plasma membrane EET receptor that activates various intracellular signaling pathways to elicit a functional response. The intracellular signaling pathways are shown in different colors to indicate that each is active in different tissues under unique conditions. There is evidence that EETs utilize cAMP and tyrosine kinase cascade signal transduction mechanisms. Activation of large-conductance Ca2+-activated K+ (BKCa) channels occurs through a G s protein coupled to the putative receptor. Whereas the cAMP-PKA, phosphatidylinositol 3-kinase (PI3K)-Akt, MAPK, and Src kinase pathways produce responses by activating gene expression, the anti-inflammatory effect produced by the IKK pathway is due to inhibition of cytokine-induced NF- B activation. HB-EGF, heparin-binding EGF-like growth factor; EGFR, EGF receptor; CREBP, cAMP response-element binding protein.

Previous studies in animal models showed the possible modulation of BP by EETs in pregnancy[209,210], some of them suggesting instead their protective role against pregnancy-induced hypertension[210]. In pregnant women, high levels of EETs were found in the fetoplacental circulation compared to that of the maternal circulation [208] and within intrauterine tissues[211,212][210] and some studies support the hypothesis that EETs play a role in BP regulation in pre-eclamptic pregnancies[207,209].

Additionally, genetic studies provided some clues concerning the possible role of these compounds in BP regulation and cardiovascular risk. In fact, human genes encoding for the major CYP isoforms codifying for the enzymes that form EETs along with *EPHX2*, the sEH gene, are highly polymorphic and a number of studies have shown that some variants are associated with a higher risk of hypertension, stroke or other major cardiovascular endpoints[214] with a possible gender-specific effect.[151,152]

Thus, a suggestive hypothesis is that omega-3 FA may additionally exert their action through the complex interaction with polymorphisms in genes encoding for CYP enzymes as was similarly hypothesized in a recent study, in which a stronger overtime reduction in BP was significantly associated with a higher Omega-3 PUFA intake, but only in subjects carrying the *CYP4F2 433VV* genotype [215] In fact, the analyzed genotype did not show an association with BP by itself in the whole population; but a significant correlation was found only when considering the interaction with Omega-3 PUFA intake, thus suggesting that Omega-3 PUFA can exert their protective effect on BP only in people carrying selected genotypes.

CYP450-derived metabolites of EPA and DHA

As previously stated, not only AA but also EPA and DHA are metabolized by COX and LOX, but particularly by CYP450 leading to the biosynthesis of a wide range of compounds that are currently attracting active research. The EPA-derived counterparts of EETs through the metabolism of CYP-epoxygenase are epoxyeicosatetraenoic acids (EEQs), whereas the DHA-derived counterparts are epoxydocosapentaenoic acids (EDPs). CYP-hydroxylase converts EPA to 19- and 20-hydroxyeicosapentaenoic acid (19- and 20-HEPE) and DHA to 22-hydroxydocosahexaenoic acid (22-HDoHe), which are the counterparts of 20-HETE [216] (Figure 5).

Epoxy metabolites of AA, EPA and DHA are further metabolized by the sEH, generating dihydroxy-fatty acids, or diols, which are the DHETs dihydroxyeicosatetraenoic acids (DiHETE) and dihydroxydocosapentaenoic acids (DHDP), respectively. This metabolic step is moreover the object of competition between AA and DHA/EPA for enzymatic binding [217].

The biological properties of the epoxides and diols derived from EPA and DHA are not yet completely understood, although substantial interest has been raised recently. In fact, the EPA and DHA epoxides have at least similar but often

stronger effects than EETs, in particular concerning their vasodilator [56],anti-inflammatory [58,217,218] and analgesic actions [217].

Animal models using canine and porcine coronary microvessels [56,219] and in rat cerebral artery [55] support the hypothesis that EEQs and EDPs act as endothelium-derived hyperpolarizing factor (EDHF) by activating Ca++-activated K+ channels and that they have a greater vasodilatory action with respect to EETs, which might be the mechanism, or at least one of the mechanisms, responsible for the BP-lowering effect of omega-3 PUFA.

Other studies in animal models stress the role of omega-3 epoxides in BP control as follows: in Angiotensin II-dependent hypertensive mice, an omega-3 rich diet in combination with the sEH inhibitor lowered BP, suggesting that omega-3 epoxides contribute to BP lowering [220]. Moreover, the same group focused attention on DHA-derived epoxides and provided some particular clues regarding 19,20-EDP as a mediator of the anti-hypertensive effect of DHA[57]. Additionally, in CYP1A1 knockout mice, the involvement of 17,18-EEQ and 19,20-EDP in BP control has been shown, suggesting vasodilation via increases in nitric oxide as a pathophysiological mechanism [221].

Moreover, the CYP/sEH-derived metabolites of omega 3 PUFA are involved in angiogenesis regulation, at minimum in retinal and tumoral vascularization as follows: omega-3 epoxide have anti-angiogenic properties[222–224], whereas sEH-derived diols may exert pro-angiogenic action [225]. Contrarily, several studies on animal models revealed that EETs were linked to angiogenesis[226–228].

Finally, EPA and DHA epoxides, along with EETs, exert anti-inflammatory actions[58,217,229]. Thus, our hypothesis maintains that the cardioprotective effects of Omega-3 FA may be explained, at least partially, by the replacement of AA-derived EETs by the more effective EPA- and DHA-derived EEQs and EDPs.

Because no reports are available concerning the biological role of 20-HEPE and 22-HDoHE in hemodynamic modulation in humans, the question of whether they share or perhaps antagonize the vasoconstrictor action of 20-HETE remains unanswered.

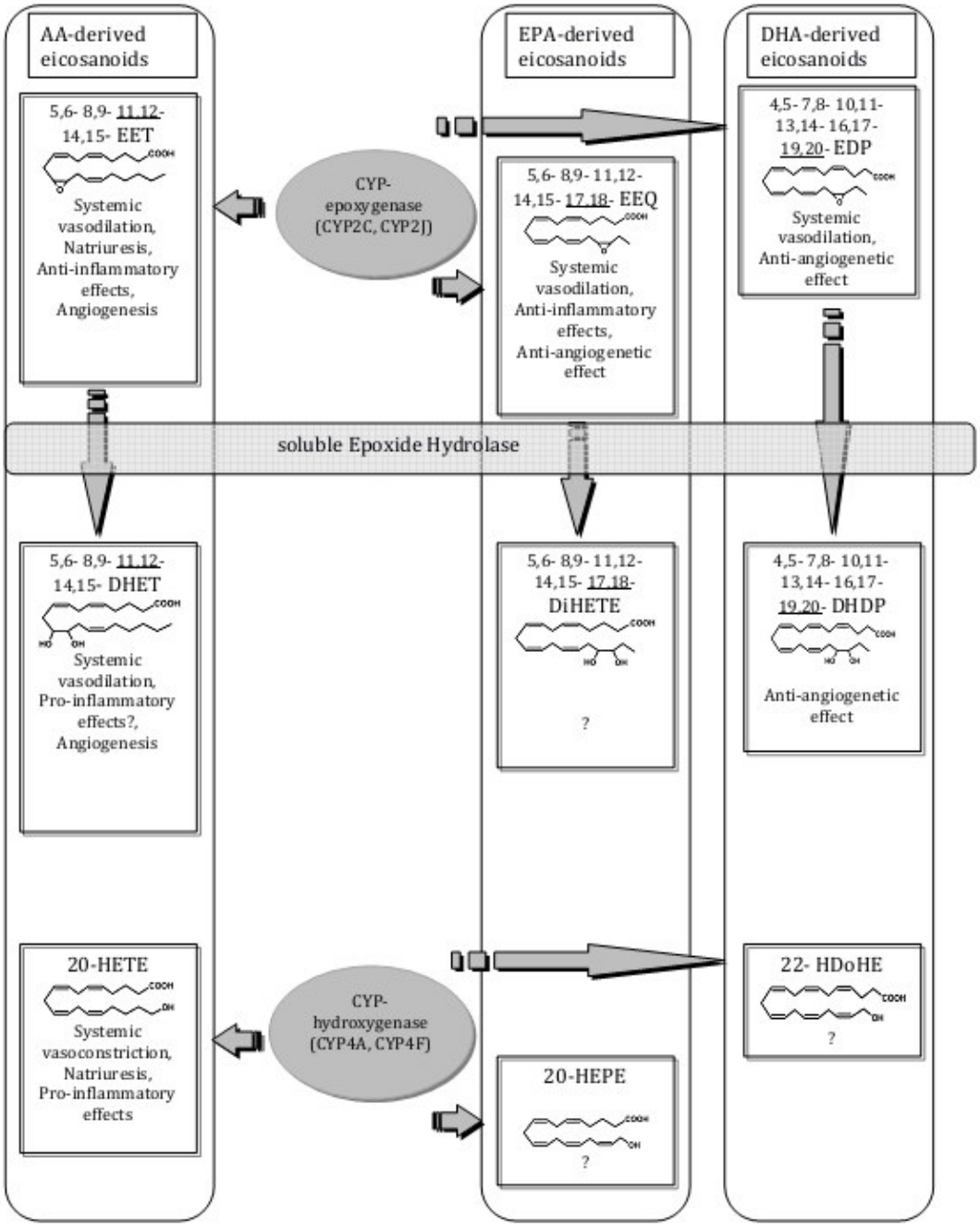


Figure 5: Reproduced with permission from Bonafini S et al. *POLM* 2017[230]

Main AA, EPA and DHA metabolites via CYP450/sEH and their principal actions.

The metabolites of AA, EPA and DHA are constituted by different isomers, which are listed in the figure; the illustrated structure refers to the underlined isomer.

AA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; EETs: Epoxyeicosatrienoic acids; 20-HETE: hydroxyeicosatetraenoic acid; EEQs: epoxyeicosatetraenoic acids; 20-HEPE: 20-hydroxyeicosapentaenoic acids; EDPs: epoxydocosapentaenoic acids; DHET: dihydroxyeicosatrienoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DHDP: dihydroxydocosapentaenoic acid; 22-HDoHE: 22-hydroxydocosahexaenoic acid

Metabolic interactions between omega-3 and omega-6 PUFA

The wide range of the metabolic pathways and biological effects of omega-3 FA makes it difficult to completely understand the clinical role of omega-3 FA and their metabolites, which remain a matter of debate. The overall picture is complicated by the possible interactions with other nutrients and dietary compounds that are far from being completely elucidated.

First, consideration should be given to the competition of omega-3 FA with omega-6 FA for the binding with several enzymes. ALA competes with LA for metabolism by delta-6 desaturase and the subsequent enzymes of the pathway, leading to the formation of EPA and AA, respectively [32]. The COX pathway catalyzes the production of prostanoids, such as the pro-thrombotic TXA2 and the anti-thrombotic PGI2, starting from AA. The counterparts derived from EPA exert more potent antiaggregatory (TXA3 and PGI3) and weaker pro-inflammatory actions (LTB5) [52].The anti-inflammatory effect of omega-3 is further explained by a novel family of compounds, the so-called protectins, resolvins and maresins [231].

Indeed, previous studies have demonstrated that many isoforms of CYP enzymes convert EPA and DHA with equal or even higher metabolic capacities compared to AA and notably, they show largely different regioselectivity [216]. In animal models, EPA and DHA have been demonstrated to replace AA in membrane phospholipids and concomitantly, clear modification exists in the balance of their metabolites with a tissue-specific effect. In rats fed with EPA + DHA supplements, the endogenous formation of EETs, EEQs and EDPs was shifted in favor of EPA and DHA metabolites with different rates in the various tissues, e.g., in the heart, the EET: EEQ: EDP ratio shifted from 87:0:13 to 27:18:55 , whereas the modification was in the same direction but with different rates in other tissues, like kidney and liver; only in brain the changes were slight[59]. Additionally, 20-HETE decreased after EPA + DHA supplementation with a concomitant increase in 20-HEPE and 22-HDoHE [59]. An interventional study in humans tested the effect of EPA + DHA supplements on the Omega-3 Index and on the balance of metabolite production. The results were consistent with the previous study on rats as follows: after increasing doses of omega-3 FA supplements for 8 weeks in 20 healthy volunteers, the Omega-3 Index increased in a time- and dose-dependent manner and a large increase was also observed in the EPA-derived metabolites via CYP epoxygenase. In particular, the ratio between the metabolites and precursors of fatty acids indicated that CYP epoxygenase was 8.6 times more efficient in EPA metabolism and 2.2 times more efficient in DHA metabolism compared to AA. Regarding the CYP-hydroxygenase pathway, no modification in 20-HETE and 22-HDoHE were observed, whereas the concentration of 20-HEPE increased 3-fold. Notably, the effect on COX and LOX remained rather weak with respect to that on the CYP epoxygenases, indicating that CYP-dependent metabolites of EPA and DHA are the putative mediators of the cardiovascular protective effects of omega-3 FA[54].

Possible confounders when evaluating the clinical effect of omega-3 FA

Notwithstanding the understanding of the biological mechanisms of action of EPA, DHA and their metabolites, uncertainty remains about their clinical efficacy. In particular, as stated previously, a discrepancy between observational and interventional studies is often evident. A possible confounder is the use of vegetables oils as controls, and often olive oil, which may in turn exert protective actions[232–234], thus partially blurring the positive effect of omega-3 FA.

Moreover, the protective effect was reported in trials using fish as the source of omega-3 FA compared to EPA+DHA supplements, although supplementation may easily provide higher doses of omega-3 FA than those supplied by fish in the diet. In our opinion, further investigation is warranted to elucidate this complex issue, that is, whether a favorable effect of omega-3 FA should be traced to these dietary fatty acids alone or to the overall sources of these compounds. Fish in particular is rich in many macro and micronutrients[235], such as vitamin D, branched chain amino acids, potassium and magnesium, that may act independently or even synergistically with omega-3 FA[236–238]. Furthermore, the intake of each individual nutrient should be considered in assessing the whole dietary pattern as follows: the intake of fish may be generally assumed to indicate healthier dietary habits and in particular, higher fish consumption is usually associated with higher vegetable and fruit intake and lower intake of meat, which are altogether often related to a higher socioeconomical status [239,240].Thus, the effects of Omega-3 FA may indeed derive from the interaction with other nutrients, such as fiber, olive oil or antioxidants, which are considered to play a role in cardiovascular risk reduction [241]. Indeed, some recent studies and meta-analyses have called attention to the role of dietary models rather than single nutrients with respect to cardiovascular risk. A systematic review of prospective studies or RCT investigating the relationship between dietary exposure and coronary heart disease identified vegetables, nuts, fish and marine Omega-3 FA, fruit, fiber and the “Mediterranean diet” as protective factors, whereas *trans* fatty acids and foods with high glycemic indexes were recognized as harmful factors [242]. A recent RCT showed that a Mediterranean diet supplemented with olive oil or nuts, which are rich in omega-6, reduced the incidence of major cardiovascular events (myocardial infarction, stroke or death from cardiovascular causes) in primary prevention [243] [244].

In our opinion, greater attention should be directed additionally to the balance between the different types of fatty acids. Undeniably, consideration should be given to the profound change in dietary patterns during the last century in Western countries whereby the omega-6: omega-3 FA balance shifted from a ratio of 1-2:1 in the Paleolithic era to the current 15-20:1 ratio [29] . This unbalance and the changes in the dietary intake of vegetables, fiber, nuts and berries are considered key factors in the development of cardiovascular disease.

Finally, the response to omega-3 FA supplements, in terms of modifications of their metabolite profile, appears to show a high grade of inter-individual variability[245], which may consequently obscure the clinical effect of omega-3 supplementation in large clinical trials.

In conclusion, evidence from observational studies, in particular, support the recommendation to enhance the intake of omega-3 FA, primarily from seafood, to reduce cardiovascular risk. We believe that their protective effects are probably mediated by an improvement in vascular function with a consequent antihypertensive effect due to the shift of CYP-derived metabolites to more potent vasodilatory agents. Persistent debate remains concerning the best source of omega-3 FA and it is not yet clarified whether the beneficial effects may be explained only by their specific biological actions or rather by their complex balance and interactions with a variety of nutrients and polymorphisms of genes implicated in their metabolic pathways.

Study 1: Omega-6 fatty acids in erythrocyte membranes are inversely associated with several features of the metabolic syndrome in a sample of obese children

Introduction Study 1

In the last few decades the lifestyle changes in western countries led to an increase in the prevalence of overweight/obesity and cardiovascular (CV) risk factors, which often accompany the body weight excess, not only in adults but also in children[109]. In particular, a central distribution of obesity is associated with a higher CV risk profile through the clustering of CV risk factors that leads to the so called metabolic syndrome (MetS). Insulin resistance often accompanies obesity and plays a pivotal pathophysiological role in the development of MetS. Non-alcoholic fatty liver disease (NAFLD) can be considered the hepatic manifestation of MetS because of the common risk factors, like central obesity, insulin resistance and dyslipidemia[246]. NAFLD by itself is associated with an increased morbidity and mortality for cardiovascular disease[247].

An unbalanced diet is a common factor that might promote several of the components of MetS and NAFLD. A hyperlipidemic diet may influence the onset and progression of obesity; however, increasing attention is paid not only to the quantity but also to the quality of fat. Indeed, epidemiological and intervention studies indicate that saturated fatty acids (SFA) might negatively affect insulin sensitivity[248] and plasma lipid profile[249]. However, randomized controlled trials (RCTs) and successive meta-analyses gave controversial results about the link between SFA and cardiovascular risk[195,250]. Anyhow, there is general agreement that dietary assumption of SFA should be lower than 10% but which is the best macronutrient to replace them remains matter of debate.

On the other hand, omega-3 polyunsaturated fatty acids (PUFA) are generally recognized as healthy nutrients. They positively affect plasma lipid profile, insulin sensitivity[251], might exert beneficial effect on blood pressure (BP)[77,230,252] and on abdominal obesity[253] and moreover can play an important role in prevention and treatment of NAFLD, even in children[246]. The increasing interest in the potential beneficial effect of omega-3 PUFA and the concern that a high omega-6/omega-3 PUFA ratio is associated with an increased cardiovascular risk led to the advisement of reducing the dietary intake of omega-6 PUFA; anyhow the role of total and single omega-6 PUFA in cardiovascular disease is not clarified yet[254].

Notably, new insights indicate a protective role of omega-6 FA, and in particular linoleic acid (LA), with respect to body fat distribution and insulin resistance[190] and total mortality[189]. A longitudinal study showed an inverse relation between an increase in serum omega-6 PUFA and the prevalence of MetS in a Finnish population[188]. Thus, further investigations are needed in order to clarify these issues in adults and even more in children. Therefore, we aimed at investigating the associations of individual CV risk factors, characterizing the MetS, with erythrocyte membrane FA, markers of the preceding two-three months intake, in a group of overweight and obese children.

Methods Study 1

Overweight and obese children were recruited consequently from October 2012 to September 2014, coming from the "Pediatric Obesity Outpatients Unit" of the University Hospital of Verona and of the "Local Health Unit n. 20" (ASL 20) of Verona. Inclusion criteria were: children and adolescents aged 5-18 years old; overweight or obesity (BMI≥90^{th} and 95^{th} percentile for sex and age, respectively)[255]. WHO reference for BMI was used for categorizing children into the overweight and obese groups[2] Exclusion criteria were: hepatic or renal chronic diseases, malignancies, diabetes mellitus, other therapies potentially affecting glucose and/or lipid metabolism; obesity secondary to genetic disorders and/or syndromes.

A subgroup of 25 children accepted to undergo a clinical follow-up and repeated the evaluation at least 6 months after the enrolment. All the families received at baseline individualized advices about a correct lifestyle including regular physical activity and a dietician proposed modification of the diet settled with the family starting from the habitual diet of the children. Children did not take any pharmacological treatment during the follow-up period. At the follow-up visit the children underwent all the same investigations as at baseline. On the basis of the change in BMI at follow-up visit compared to the first evaluation, regardless to the change in BMI percentile, the children were classified as good (decreased BMI) or poor (increased BMI) compliers to the suggested lifestyle advices.

STUDY DESIGN

The study was conducted according to a cross-sectional observational design for the first part and according to a longitudinal observational study for the follow-up of the subgroup of 25 children. The study was approved by the Ethical Committee of Verona (CE n. 2218), and written informed consent was obtained from each participant's parents.

ASSESSMENTS

Each child was evaluated in a single occasion, between 8 and 9 a.m. A questionnaire was administered to the patients and to the parents, dealing with medical history, family history, physiological and pathological information and use of drugs. Then, the participants underwent a physical examination. They were advised not to engage in strenuous exercise and to avoid consuming caffeine containing beverages within 12 hours preceding the vascular studies.

During the visit, blood pressure was measured with a semiautomatic oscillometric device (TM-2551, A&D instruments Ltd, Abingdon Oxford, UK) for 3 times, 3 minutes apart with the patient lying supine for at least 10 minutes before the first measurement in a room with controlled temperature (22-24°C). The mean value of the 3 clinostatic measurements were calculated and considered for z-score and percentile calculation. Afterward, BP levels were confirmed by a measurement in the sitting position by the oscillometric device and by auscultatory method. Ambulatory blood pressure measurement was recorded by an oscillometric device (Spacelabs 90217; Spacelabs Inc., Issaquah, Whashington, USA), which measured BP every 15 minutes during the day and every 30 minutes during the night. Children and parents recorded physical activities, resting and sleeping time and symptoms on a dedicated diary. After recording, the daytime and nighttime periods (set to default at 0700 and 2200 h, respectively) were adapted to "real"

awake and sleep times according to what was declared in the diary of activity, as previously indicated[256].
All of the values derived from BP measurements were transformed in z-score and percentile, according to normative values[257,258]. The 95th of office and ambulatory BP measurements was used as cut-off for hypertension, according to current European guidelines[26].
Body weight, height, and waist and hip circumferences were measured with the patient wearing light clothes. Body weight was measured by a calibrated balance and height by a calibrated stadiometer[259].
Waist circumference was transformed in z-score and percentile according to normative values[260].
Metabolic Syndrome definition
In an exploratory analysis, we defined the MetS according to the diagnostic criteria suggested by the International Diabetes Federation[261]. Anyhow we decided to extend the diagnosis to all age categories and replace the suggested cut-off point of BP with the 90th percentile for sex and age of SBP and DBP, according to the normative values[258] and to the definition of pre-hypertension/hypertension indicated by the current guidelines[26].
Laboratory measurements
Blood samples were collected after an overnight fast. Laboratory measurements, including fasting plasma glucose, insulin, total cholesterol, HDL-cholesterol, triglycerides, AST, ALT and GGT were measured using standardized methods. Insulin resistance has been estimated with HOMA Index (HOMA-IR), which was calculated by Matthews formula (fasting insulin (mU/mL)*fasting glucose (mmol/L)/22.5)[262].
Fatty liver index (FLI), derived from an algorithm based on BMI, waist circumference, triglycerides and GGT, were calculated as previously described [263]
Red blood cell membrane fatty acids measurement
EDTA-blood tubes were centrifuged, plasma and buffy coat taken off, and erythrocytes frozen at -80°C until analysis. Erythrocyte fatty acid composition was analyzed using the HS-Omega-3 Index® methodology as previously described[264]. Fatty acid methyl esters were generated from erythrocytes by acid transesterification and analyzed by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu, Duisburg, Germany) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA) using hydrogen as carrier gas. Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of erythrocytes. A total of 26 fatty acids were identified and quantified.
Results are given as percentage of total identified fatty acids after response factor correction. The coefficient of variation for EPA plus DHA and for most other fatty acids was 4%. Analyses were quality-controlled according to DIN ISO 15189.
Estimation of Δ^9, Δ^6 and Δ^5 desaturase activity
Δ^9, Δ^6 and Δ^5 desaturase are enzymes responsible for the endogenous formation of monounsaturated and polyunsaturated FA and their activity has been associated with insulin-glucose homeostasis and with central obesity[265].
We estimated the desaturase activity as the ratio of product to precursor of individual red blood cell membrane FA as follows: Δ^9-desaturase (SCD) =

C16:1n-7/C16:0 and C18:n-9/C18:0 (they will be referred to as SCD-16 and SCD-18, respectively); Δ^6-desaturase (D6D) = C18:3n-6/C18:2n-6 and Δ^5-desaturase (D5D) = C20:4n-6/C20:3n-6.

Hepatic ultrasonography

Children underwent abdomen ultrasonography (US) using a convex probe (ACUSON S2000TM system, Siemens, Erlanger, Germany). The presence of significant liver steatosis was defined by an experience sonographer.

STATISTICS

Data are presented as median and range unless otherwise stated. The normal distribution of each variable was evaluated by the Kolmogorov-Smirnov test. Differences in the measured parameters between groups (MetS presence, steatosis, gender, pubertal state) were analyzed by T-test or Mann Whitney U test, as appropriate, at univariate analysis, and by unconditional logistic regression models at multivariate analysis. Unless otherwise specified, covariates included in the multivariate models were age, sex, BMI percentile and the features associated, at univariate analysis, with MetS presence, steatosis, gender, and pubertal state, respectively.

Since MetS-associated features were non-normally distributed, bivariate correlations were estimated by the non-parametric Spearman correlation coefficient (r_S). After log-transformation of MetS-associated features, general linear models were applied to assess the association of FA with MetS-associated features, after adjustment for age, sex and BMI.

In longitudinal study, significant variations in children features between baseline and follow-up were evaluated with the Wilcoxon signed rank test.

Two-tailed tests with a $p<0.05$ were considered statistically significant in the main analysis. In order to take into account the multiple comparisons, along with original p-values, the false discovery rate (FDR) adjusted p-values were also calculated and reported in the tables, where appropriate. The analyses were performed with Statistical Analysis System (SAS) Software, version 9.2.

Results Study 1

General characteristics at baseline

Seventy patients (40 males and 30 females) were included in the study and 25 children completed the follow-up (11 males and 14 females). Mean age was 11.5 ± 2.5 years; mean BMI was 29.4 ± 4.4 Kg/m^2, which in all cases was higher than the 90th percentile, as for inclusion criteria. Five children (7.1%) fulfilled the diagnostic criteria of MetS. Sixty-three children underwent abdomen ultrasonography (US) and in 34 (53%) children hepatic steatosis was detected.

Blood samples for laboratory measurements and FA analysis were obtained from all children.

Omega-3 Index was 4.7 ± 0.8 %; mean value of ALA was 0.08 ± 0.03 %, and LA was 12.0 ± 1.6 %.

Anthropometric, clinical and biochemical characteristics of the children are listed in **Table 1**, and the FA contents of erythrocytes in **Supplemental Table 1**.

Correlations between red blood cell membrane FA and features of MetS
Omega-3 PUFA, as well as EPA and DHA, correlated directly with waist circumference; moreover, EPA showed direct correlations with insulin, HOMA-IR and with FLI (**Table 2**).
To the contrary, total omega-6 PUFAs (LA+ gamma-linolenic acid (GLA) + dihomo-gamma-linolenic acid (DGLA) + AA + docosatetraenoic acid (DTA) + eicosadienoic acid + C22:5n6) was inversely correlated with several anthropometric and laboratory measurements related to MetS; within omega-6 PUFA, AA in particular was associated with almost the same characteristics as the class of FA (Table 2 and **Figure 1**). Conversely, gamma-linolenic acid (GLA) showed a direct correlation with some features of Mets, especially with total cholesterol, triglycerides, fasting insulin, HOMA-IR and FLI.
Total content of SFA was directly correlated with several features of the MetS, within SFA, palmitic acid (PA) in particular directly correlated with several individual characteristics (Table 2 and **Figure 2**).
Omega-9 FAs and *trans*-FAs, when considered either the single FA or their sum, did not show significant correlations with anthropometric, clinical and laboratory parameters of MetS.

Correlations between red blood cell membrane FA and features of NAFLD
As for the correlation with individual components of the MetS, omega-6 PUFA and in particular AA showed an inverse correlation with FLI and transaminases, whereas SFA, and particularly PA, resulted directly correlated (see Table 2).

Correlations between desaturase activity and features of MetS
D6D activity showed direct correlations with waist circumference (r_S = 0.249), triglycerides (r_S = 0.370), fasting insulin (r_S = 0.404), HOMA-IR (r_S = 0.423) and with FLI (r_S = 0.402).
D5D activity was inversely correlated to triglycerides (r_S = -0.401), and FLI (r_S = -0.319). (Supplemental Figure S1)
No significant differences were found in desaturase activity according to gender, pubertal status and presence of hepatic steatosis.

Regressions
After adjustment for sex and age all the correlations shown in Table 2 remained significant. When also BMI was included in the regression most associations remained significant, especially the ones with laboratory parameters (see Table 2).

Analysis by subgroups
When comparing children with and without MetS (n: 5 and 53, respectively), we observed a significantly higher SBP, triglycerides, PA and D6D and lower HDL-cholestreol, AA and omega-6 in patients with MetS, after adjustment by age and BMI. Otherwise, no feature was associated with liver steatosis at multivariate analysis. The characteristics of the subgroups are detailed in **Supplemental Table 2** and **Supplemental Table 3**.

Follow-up

Twenty-five children (11 males and 14 females) completed the follow up. The mean time of follow-up was 16.2 ± 9.2 months. The main characteristics at the follow-up visit are detailed in **Table 3**.

We found a significant reduction of BMI percentile, 24-h SBP and DBP percentile, total cholesterol and tryglicerides in children at follow up compared to baseline and a significant increase in HDL levels (Table 3).

We found that the basal values of AA were inversely correlated with the change over time of waist circumference (r_S =-0.506) and office SBP (r_S =-0.710).

Discussion Study 1

The main result of our study is the evidence that omega-6 FA, and in particular AA, are inversely associated, whereas SFA directly, with many components of MetS in obese children. Even if the observational design of the study does not allow to proof a causal link, our results could suggest, on one side, a protective role of omega-6 FA and, on the other side, a harmful effect of SFA with respect to cardiovascular risk factors and NAFLD.

Anyhow, these associations of opposite sign could simply reflect a healthier dietary habit, with a preferential intake of omega-6 PUFA with respect to SFA. Therefore, the relative increase in omega-6 PUFA could not be, or not only, protective *per se* but instead be a marker of the reduction of other potentially harmful components of the diet, like saturated FA. Indeed, a diet rich in polyunsaturated FA is often associated to healthier dietary pattern, which may involve also other macronutrients[240].

Despite an increasing expectation from a beneficial effect on cardiovascular risk profile and NAFLD in children by omega-3 PUFA, and in particular EPA and DHA[246], we did not find any significant beneficial association between either EPA or DHA and clinical/laboratory characteristics of our population. To the contrary, we found a direct association of EPA with some characteristics of the MetS, in particular insulin and HOMA-IR. The effect of omega-3 PUFA on glucose metabolism is not clearly defined and trials in humans did not give unequivocal results[266,267]. In our sample, that at baseline was taking a free diet without any supplement, the level of omega-3 PUFA was very low, far below the 8% threshold suggested for CV protection[268] and this could also be a confounding element.

We choose red blood cell membrane FA as a biomarker of dietary intake of FA because of the stability of their values, especially of essential FA, which reflect the mean dietary intake in the preceding few months[269], and because it is more reliable compared to dietary self-report or questionnaire, especially in children.

Our results confirm that total amount of SFA is associated with an unfavourable cardiovascular risk profile in obese children and, within this family of FA, palmitic acid show to be related to almost all the characteristics of the MetS. It has been already shown that palmitic acid is one of the most abundant circulating FA in obese children[270] and elevated palmitic acid levels can affect insulin homeostasis, which is the principal etiologic driver of the metabolic abnormalities clustering in the MetS. In particular, *in vitro* studies reported an impairment in insulin secretion in murine[271] and human[272] β-cell lines due to palmitic acid, generally referred to as lipotoxicity. A few *in vivo* studies support this results: one

study in adults reported an altered postprandial insulin secretion and sensitivity in response to a high dietary intake of palmitic acid[273], another study in children and adolescents showed that obese subjects with higher circulating levels of palmitic acid have an increased and delayed insulin secretion[274]. Furthermore, it has been shown that palmitic acid in plasma triglycerides was higher in abdominally obese adults as compared to the controls without central obesity and this FA profile was directly correlated to HOMA-IR[275].

As already stated above, our results might indicate also a protective role of omega-6 PUFA, and in particular of AA, with respect to several cardiovascular risk factors. Previous studies investigating the effect of omega-6 in cardiovascular disease gave conflicting results, some suggesting no association between omega-6 PUFA and the risk of hypertension[80] and other that the replacement of SFA with omega-6 PUFA is not associated with vascular and endothelial function but might improve BP[185]. Indeed, dietary intake of LA may contribute to prevention and control of elevated blood pressure[191]. Thus, although omega-6 PUFA have been for a long time counter-posed to the beneficial omega-3 PUFA, recent insight supports their potential cardiovascular protective effect[189].

Also when considering specifically AA, the omega-6 which drive the observed association, available data show contrasting results. A higher amount of AA was found in adipose tissue in metabolically unhealthy obese adults compared to metabolically healthy control[186]. Then, in healthy children, AA, measured in adipose tissue and in skeletal muscle cells, was directly correlated to fasting insulin and HOMA-IR[199]. Nevertheless, some studies indicated a protective effect of AA, especially when measured in red blood cell membranes, on glucose-insulin homeostasis[192,193], even if some observations suggest a beneficial effect in subject with low-normal insulin sensitivity but not in highly insulin-sensitive individuals[194]. Interestingly, our exploratory analyses at follow up confirm a possible protective effect of AA on body weight and BP control also over time. It is not clear why GLA, in contrast to the other omega-6 FA, is directly correlated with a poorer metabolic profile. On one side, previous studies have indicated that GLA leads to the production of anti-inflammatory compounds, but GLA may also inhibit the metabolism of AA, at least in some types of cells[276]. Moreover GLA is yielded from LA by D6D and leads to the production of DGLA, which is the substrate of D5D. Therefore the association of GLA with the clinical parameters could also reflect the activity of the desaturase enzymes.

In fact, also the role of fatty acid desaturase (SCD, D6D and D5D) has been linked to visceral obesity[265] and insulin resistance, suggesting an increased SCD and D6D and a decreased D5D activity in subjects with impaired insulin sensitivity and related disorders[277–279]. Our results support the findings of previous studies in adults, showing that the estimated D6D activity is directly related to a poorer metabolic profile, whereas D5D activity is inversely related, in our group of obese children.

In addition, omega-6 PUFA, SFA and D6D showed to be correlated with waist circumference, marker of central adiposity, supporting the association of dietary fat with the body fat distribution and the cardiometabolic profile, even if the association disappears after further adjustment for BMI.[280,281]

Furthermore, the associations of the different FA and FA families with a possible hepatic involvement, configuring the NAFLD, beside the correlation with the individual components of the Mets, supports the hypothesis that NAFLD represents a continuum with MetS subtended by insulin resistance as the common pathophysiological background[282].

Our study has limitations: the sample size is relatively low, which can primarily expose to a problem of statistical power. Nevertheless, we were able to detect some meaningful associations between lipid composition of erythrocyte membrane and many parameters of the MetS. These results need to be confirmed in other studies analysing also samples of children of different ages and body size, including non-obese children. Moreover, data coming from other ethnic groups, which often have different dietary habits, could help a better understanding of these associations. Then, it remains to be clarified if the putative beneficial effect of omega-6 PUFA is specific for obese children and/or viewable only when omega-3 PUFA are under a certain threshold.

Moreover, as above mentioned, the observational design of the study do not allow any causative link and the small number of children included in the follow-up limits the interpretation of these data. Further studies are needed to confirm our results.

Lastly, we lack data about the total food intake of fatty acids and the amount of fatty acids and the other macronutrients in the diet, even if dietary tools to collect these data are often inaccurate, especially in children. Anyhow, it is possible to speculate that data from dietary assessment, like food frequency questionnaire or food diary, together with the measurement of fatty acids in red blood cell membranes, could have led to discover weather, on one side, the balance between different fatty acids or with the other macronutrients, on the other side if the entire family or a single FA play a role in metabolic modulation.

Strengths of our study regards the exploration of a topic in children, in which only a few studies are available, the in-depth characterization of the children's clinical and laboratory characteristics of the MetS, finally the use of the gold standard technique for the assessment of fatty acids in red blood cell membrane. Unfortunately, the longitudinal part of the study has collected only 1/3 of the initial sample but still allowed to confirm at least some association finally strengthening our conclusions.

Further studies, including intervention trials, are needed in order to better understand the actions of the different FA on the single components of the MetS even in children.

In conclusion, the present study shows an association between FA, reflecting their dietary intake, and MetS, which supports the hypothesis that the quality of fat intake, beyond the quantity, can influence the metabolic profile in obese children. Our findings agree with the current dietary recommendation to reduce the intake of SFA and support a possible beneficial effect of polyunsaturated FA intake, especially omega-6 PUFA. The level of omega-3 PUFA in our sample of obese children, is extremely low so that its putative beneficial effect could have not been detectable.

Table 1. General characteristics of the obese children at baseline.

Variable	Males (n=40) Median (range)	Females (n=30) Median (range)	p-value[1]	p-value[2]	Pre-pubertal (n=38) Median (range)	Post-pubertal (n=32) Median (range)	p-value[1]	p-value[2]
BMI (Kg/m^2)	28.5 (23.1-42.7)	29.3 (24.5-40.6)	0.55	-	28.2 (23.9-38.4)	29.9 (23.1-42.7)	0.06	-
Percentile BMI	98.3 (93.5-99.8)	98.7 (90.2-99.9)	0.50	0.36	98.9 (94.9-99.9)	98.0 (90.2-99.8)	**0.03**	0.56
Waist (cm)	96.0 (79.0-122.0)	95.5 (82.0-119.0)	0.92	-	91.0 (79.0-122.0)	97.0 (83.0-119.0)	**0.02**	**-**
Percentile Waist	97.1 (91.9-99.2)	98.2 (90.7-100.0)	**0.04**	0.11	97.9 (92.1-100.0)	97.0 (90.7-99.1)	**0.01**	0.09
O-SBP (mmHg)	119.0 (102.0-163.0)	115 (105-143)	0.31	-	116.0 (102.0-163.0)	122.0 (107.7-152.3)	**0.04**	**0.009**
Percentile O-SBP	85.1 (44.7-100.00)	78.2 (45.4-99.9)	0.92	-	80.8 (44.7-100.0)	82.8 (45.4-100.0)	1.00	-
O-DBP (mmHg)	68.3 (52.0-88.3)	66.5 (56.7-83.7)	0.42	-	66.7 (56.7-88.3)	67.5 (52.0-83.7)	0.46	-
Percentile O-DBP	65.7 (6.6-98.0)	65.4 (23.1-96.3)	0.93	-	66.1 (19.1-98.0)	63.2 (6.6-96.3)	0.29	-
24h-SBP (mmHg)	118.0 (107.0-136.0)	112.0 (100.0-130.0)	**0.001***	0.51	115.0 (102.0-136.0)	117.0 (100.0-130.0)	0.64	-
Percentile 24h-SBP	74.0 (26.3-99.7)	51.7 (4.6-99.6)	0.10	-	75.6 (21.4-99.7)	51.1 (4.6-99.6)	**0.01**	0.08
24h-DBP (mmHg)	68.0 (58.0-79.0)	64.0 (55.0-74.0)	**0.001***	**0.02**	67.0 (55.0-79.0)	65.0 (58.0-78.0)	0.16	-
Percentile 24h-DBP	56.6 (3.7-99.0)	34.5 (2.1-94.8)	**0.005 ***	-	52.8 (2.1-99.0)	35.1 (3.7-98.4)	0.09	-
TC (mg/dL)	160.0 (106.0-242.0)	162.0 (93.0-216.0)	0.96	-	163.0 (105.0-213.0)	153.0 (93.0-242.0)	0.75	-
HDL (mg/dL)	49.0 (30.0-77.0)	52.0 (37.0-81.0)	0.07	-	50.0 (33.0-77.0)	49.0 (30.0-81.0)	0.91	-
TG (mg/dL)	79.0 (28.0-285.0)	73.0 (34.0-143.0)	0.43	-	85.0 (28.0-285.0)	69.0 (34.0-208.0)	0.10	-
AST (U/L)	27.5 (15.0-74.0)	21.5 (15.0-36.0)	**0.004***	0.08	27.0 (15.0-74.0)	24.0 (15.0-59.0)	0.14	-
ALT (U/L)	28.5 (13.0-189.0)	19.0 (13.0-61.0)	**0.01**	0.61	23.0 (13.0-189.0)	24.5 (13.0-157.0)	0.61	-
GGT (U/L)	16.0 (4.0-79.0)	13.0 (6.0-28.0)	**0.03**	0.90	14.0 (4.0-79.0)	14.0 (4.0-47.0)	0.97	-
Fast. GLU (mg/dl)	88.0 (81.0-117.0)	85.0 (70.0-99.0)	**0.01**	**0.02**	88.0 (75.0-117.0)	86.0 (70.0-108.0)	0.35	-
Fast. INS (uU/ml)	19.2 (3.0-62.5)	17.8 (5.3-43.4)	0.79	-	17.8 (3.0-62.5)	20.7 (6.8-49.8)	0.28	-
HOMA-IR	3.9 (0.6-18.0)	4.1 (0.0-8.0)	0.55	-	4.0 (0.6-18.0)	4.0 (0.0-12.4)	0.74	-
FLI	31.9 (6.3-98.7)	31.5 (7.3-91.7)	0.46	-	25.3 (6.3-95.6)	33.4 (7.3-98.7)	0.30	-

FLI: fatty liver index; HDL: HDL cholesterol; O-SBP: Office systolic blood pressure; O-DBP: Office diastolic blood pressure; TC: total cholesterol; TG: Triglycerides; Waist: Waist Circumference.

[1] Wilcoxon-Mann-Whitney U Test[2] Multivariate model includes variables significantly associated with sex and pubertal status, respectively, at univariate analysis plus age, sex and percentile BMI, where appropriate. * $p<0.05$ after False-Discovery-Rate adjustment for multiple testing

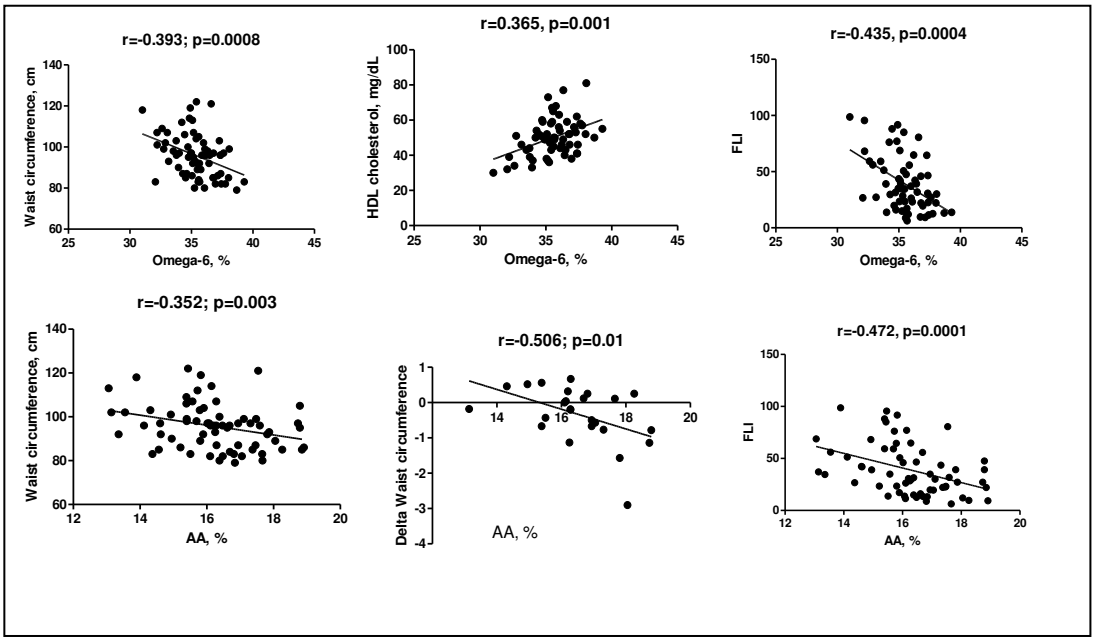
***Table 2**. Correlations between different erythrocytes membrane FA and clinical and laboratory features of MetS and NAFLD.*

	Waist	Z-score waist	24h-SBP	Z-score 24h-SBP	24h-DBP	Z-score 24h-DBP	Total chol.	HDL chol.	Triglycerides	Insulin	Glucose	HOMA-IR	ALT	GGT	FLI
Omega-6	**-0.393°**	-0.055	-0.218	0.054	-0.023	0.034	-0.005	**0.365^**	**-0.322^**	**-0.377^**	-0.037	**-0.338***	**-0.287***	**-0.256***	**-0.435°**
LA	-0.167	-0.125	0.097	0.224	0.171	0.199	0.061	**0.327^**	-0.087	-0.050	-0.033	-0.103	ns-0.154	-0.228	-0.194
AA	**-0.352^**	-0.058	**-0.313***	-0.105	-0.212	-0.163	-0.176	0.129	**-0.379^**	**-0.337^**	-0.056	-0.265	**-0.331^**	-0.221	**-0.472°**
GLA	0.211	0.158	0.137	0.102	0.191	0.168	**0.286***	-0.069	**0.368^**	**0.408°**	0.164	**0.375^**	0.141	0.200	**0.351^**
DGLA	0.057	0.072	0.150	0.090	0.131	0.134	0.226	-0.092	**0.384^**	0.196	0.233	0.240	0.151	0.158	0.218
SFA	**0.237***	0.060	0.196	0.057	0.153	0.106	0.017	**-0.265***	**0.262***	0.203	-0.084	0.258	**0.284***	**0.400^**	**0.479°**
PA	**0.354^**	0.119	0.132	-0.046	0.054	0.000	0.180	-0.222	**0.400°**	**0.287***	-0.049	**0.335***	0.239	**0.339^**	**0.515°**
Omega-3	**0.299***	0.039	0.048	-0.158	-0.114	-0.150	-0.012	-0.025	-0.156	0.105	-0.036	0.027	-0.001	0.007	0.109
ALA	0.008	0.117	-0.073	0.037	0.048	0.057	0.124	-0.065	**0.300***	0.241	**0.256***	0.230	0.025	0.125	0.126
EPA	**0.390°**	**0.295***	0.151	0.010	-0.072	-0.097	0.010	-0.133	0.155	**0.292***	0.152	**0.346***	0.094	0.001	**0.312***
DHA	**0.279***	-0.042	0.022	-0.193	-0.138	-0.172	-0.026	-0.049	-0.169	0.074	-0.079	-0.019	-0.017	-0.012	0.089
D6D	**0.249***	0.201	0.147	0.091	0.176	0.150	0.240	-0.161	**0.370^**	**0.404^**	0.165	**0.423^**	0.162	0.238	**0.402^**
D5D	-0.155	-0.077	-0.202	-0.102	-0.171	-0.158	-0.239	0.109	**-0.401°**	-0.229	-0.217	-0.239	-0.182	-0.164	**-0.319***

*: p<0.05; ^: p<0.01, still significant after False-Discovery-Rate adjustment for multiple testing; °: p<0.001, still significant after False-Discovery-Rate adjustment for multiple testing

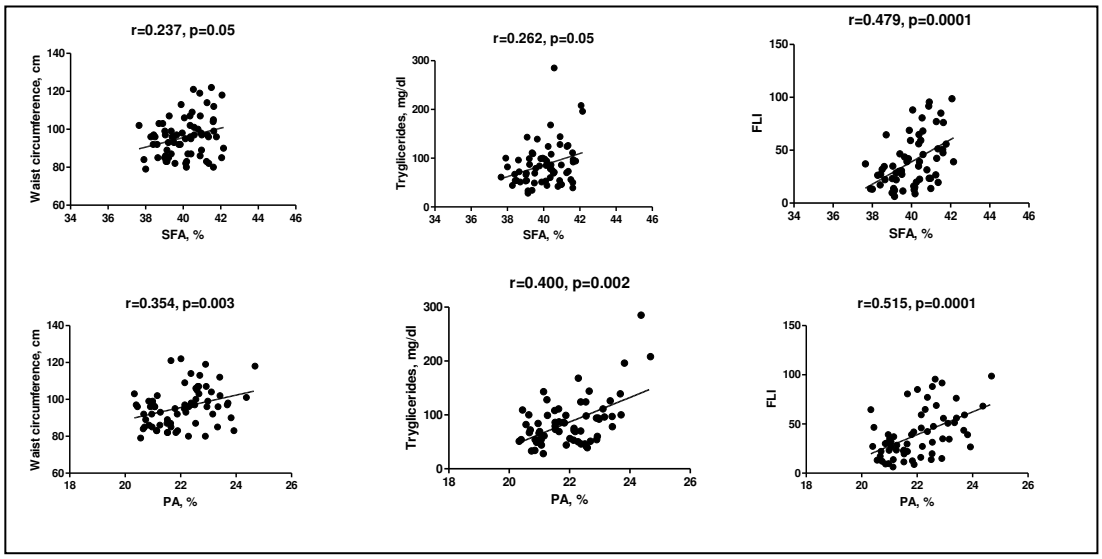
In the table are reported the r_S values of the correlations. The underlined correlations are significant after adjustment for sex, age and BMI. AA: arachidonic acid; ALA: alpha-Linolenic; ALT: alanine aminotransferase; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; D5D: delta-5 desaturase; D6D: delta-6 destaurase; EPA: Eicosapentaenoic acid ; FLI: fatty liver index; GGT: gamma-glutamyltransferase; GLA: gamma-Linolenic acid; HOMA-IR: Homeostatic model assessment – insulin resistance; LA: Linoleic acid; omega-3 PUFA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and (DHA) ; omega-6 PUFA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6, saturated FA (SFA) are calcuated as the sum of C14:0, Palmitic acid, Stearinic acid and Lignocerinic acid; PA: palmitic acid; Waist: waist circumference

Figure 1. Correlations of total omega-6 FA and AA with some features of MetS and NAFLD



Omega-6 FA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA), Eicosadienoic acid and C22:5 ω6; AA: Arachidonic acid; FLI: fatty liver index; Delta waist circumference was calculated as (Waist circumference at follow-up – basal waist circumference) / follow-up period (months).

Figure 2. Correlations of SFA and PA with some features of MetS and NAFLD



Saturated FA (SFA) are calcuated as the sum of C14:0, Palmitic acid, Stearinic acid and Lignocerinic acid; PA: palmitic acid; NAFLD: non alcoholic fatty liver disease; FLI: fatty liver index.

Table 3. Median difference between follow-up and baseline of general characteristics in the whole sample of obese children and according to decrease (good complier) or increase (poor complier) of BMI with respect to baseline.

Variable	All children (N=25) Median difference FU-BL	All children p-value[1]	Good compliers (N=15) Median difference FU-BL	Good compliers p-value[1]	Poor compliers (N=10) Median difference FU-BL	Poor compliers p-value[1]
BMI (Kg/m^2)	-0.4	0.11	-2.2	**<0.0001**	0.9	**0.004**
Percentile BMI	-1.2	**<0.0001***	-2.7	**<0.0001**	-0.5	0.19
Waist (cm)	-3.0	0.15	-4.0	**0.04**	2.5	0.82
Percentile Waist.	-0.5	0.18	-1.0	**0.03**	0.5	0.56
O-SBP (mmHg)	2.3	0.46	-2.3	0.55	9.0	0.13
Percentile O-SBP	-2.4	0.23	-8.7	**0.04**	4.9	0.56
O-DBP (mmHg)	2.0	0.38	-1.7	0.77	4.3	0.14
Percentile O-DBP	2.8	0.65	-5.1	0.56	10.5	0.19
24h-SBP (mmHg)	-0.5	0.39	-1.0	0.55	-0.5	0.57
Percentile 24h-SBP	-13.4	**0.006**	-16.3	**0.02**	-1.9	0.38
24h-DBP (mmHg)	0.0	0.26	0.0	0.66	-1.0	0.20
Percentile 24h-DBP	-4.4	**0.009**	-2.7	0.15	-7.0	**0.02**
Tot. CHOL (mg/dL)	-9.0	**0.03**	-9.0	0.06	-9.0	0.26
HDL (mg/dL)	3.0	**0.03**	3.5	0.06	1.0	0.44
TRI (mg/dL)	-13.5	**0.02**	-13.0	**0.04**	-14.0	0.25
ALT (U/L)	-3.0	0.11	-3.0	0.12	-2.0	0.67
GGT (U/L)	-2.0	0.24	-3.0	**0.04**	-1.0	0.75
Glucose (mg/dl)	-2.0	0.18	-2.0	0.09	0.0	1.00
Insulin (uU/ml)	-3.8	0.07	-4.7	0.19	-3.8	0.30
HOMA-IR	-0.8	0.25	-0.9	0.21	0.7	0.81

ALT: alanine aminotransferase; BL: Baseline; BMI: Body Mass Index; FU: Follow-Up; GGT: gamma-glutamyltransferase; HDL: HDL cholesterol; HOMA-IR: Homeostatic model assessment – insulin resistance; O-SBP: Office systolic blood pressure; O-DBP: Office diastolic blood pressure; Tot. CHOL: total cholesterol; TRI: Triglicerides; Waist:Waist Circumference.

Median difference FU-BL was calculated as the difference between the median value of the variable at follow-up (FU) and that at baseline (BL).

[1] Wilcoxon Signed Rank Test for paired data

* $p<0.05$ after False-Discovery-Rate adjustment for multiple testing

Supplemental Material Study 1

Supplemental Table S1. Fatty Acids Composition of erythrocytes plasma membrane in the obese children at baseline.

Variable	Males (n=40) Median (range)	Females (n=30) Median (range)	Univ. p-value[1]	Multiv. p-value[2]	Pre-pubertal (n=38) Median (range)	Post-pubertal (n=32) Median (range)	Univ. p-value[1]	Multiv. p-value[2]
PA (%)	22.2 (20.4-24.7)	21.8 (20.3-23.7)	0.29	-	21.9 (20.4-24.4)	22.4 (20.3-24.7)	0.36	-
LA (%)	11.7 (7.6-16.2)	11.9 (8.8-17.1)	0.38	-	12.2 (10.0-17.1)	11.5 (7.6-15.9)	0.10	-
GLA (%)	0.1 (0.0-0.3)	0.1 (0.1-0.3)	0.33	-	0.1 (0.0-0.3)	0.1 (0.0-0.3)	0.19	-
DGLA (%)	2.0 (1.6-3.2)	2.0 (1.3-2.7)	0.84	-	2.0 (1.6-3.2)	2.0 (1.3-2.8)	0.93	-
AA (%)	16.0 (13.1-18.7)	16.4 (13.1-18.9)	0.12	-	16.2 (13.1-18.9)	16.2 (13.1-18.8)	0.51	-
ALA (%)	0.1 (0.0-0.2)	0.1 (0.0-0.2)	0.56	-	0.1 (0.0-0.2)	0.1 (0.0-0.2)	**0.04**	0.10
EPA (%)	0.4 (0.2-0.7)	0.4 (0.2-0.8)	0.40	-	0.4 (0.2-0.7)	0.4 (0.2-0.8)	0.09	-
DHA (%)	4.3 (2.6-6.1)	4.4 (2.8-6.0)	0.38	-	3.9 (2.6-5.9)	4.7 (2.9-6.1)	**<0.0001***	**0.001**
Omega3-Index (%)	4.6 (2.9-6.6)	4.6 (3.0-6.6)	0.36	-	4.3 (2.9-6.3)	5.1 (3.5-6.6)	**<0.0001***	**0.001**
Omega-3 FA (%)	6.3 (4.3-9.0)	6.3 (4.5-8.7)	0.61	-	6.1 (4.3-8.5)	6.9 (5.2-9)	**0.0004***	**0.002**
Omega-6 FA (%)	35.4 (31.0-38.7)	35.6 (33.0-39.3)	0.08	-	36.1 (32.2-39.3)	35.0 (31.0-38.1)	**0.02**	**0.002**
Omega-9 (%)	16.4 (14.9-19.8)	16.4 (14.0-19.4)	0.55	-	16.3 (14.4-19.4)	16.7 (14.0-19.8)	0.13	-
SFA (%)	40.5 (38.0-42.2)	39.7 (37.7-41.6)	**0.04**	0.21	40.2 (38.0-42.2)	40.1 (37.7-42.1)	0.43	-
D6D	0.009 (0.004-0.02)	0.008(0.004-0.02)	0.31	-	0.009 (0.004-0.02)	0.008 (0.005-0.02)	0.41	-
D5D	7.6 (4.6-11.4)	8.1(5.7-13.6)	0.53	-	7.9 (4.6-11.1)	7.5 (5.1-13.6)	0.90	-

AA: Arachidonic acid; ALA: alpha-Linolenic.PA: palmitic acid; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; D5D: Δ^5-desaturase = C20:4n-6/C20:3n-6; D6D: Δ^6-desaturase = C18:3n-6/C18:2n-6; GLA: gamma-Linolenic acid; Omega-3 Index: sum of Omega 3 fatty acids (EPA and DHA); LA: Linoleic acid; saturated FA (SFA) are calcuated as the sum of C14:0, Palmitic acid, Stearinic acid and Lignocerinic acid; Omega-3 FA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and (DHA); Omega-6 FA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6

[1] T-test or Wilcoxon-Mann-Whitney U Test, as appropriate

[2] Multivariate p-values were calculated for fatty acids significantly associated with sex and puberal status, respectively, at univariate analysis. Covariates included general characteristics significantly associated with sex and pubertal status, respectively, at multivariate analysis (see Table 1a) plus age, sex and percentile BMI, where appropriate

* $p<0.05$ after False-Discovery-Rate adjustment for multiple testing

Supplemental Table S2. General characteristic of the obese children divided according to the presence of liver steatosis and metabolic syndrome at baseline.

Variable	Steatosis (n=34) Median (range)	Not Steatosis (n=29) Median (range)	p-value[1]	p-value[2]	MetS (n= 5) Median (range)	Not MetS (n=53) Median (range)	p-value[1]	p-value[2]
BMI (Kg/m^2)	29.5 (23.9-42.7)	28.2 (23.1-37.2)	0.25	-	25.5 (23.1-42.7)	28.7 (23.9-40.6)	0.49	-
Percentile BMI	98.8 (93.5-99.8)	98.2 (94.3-99.9)	0.33	0.16	97.5 (96.9-99.8)	98.6 (90.2-99.9)	0.38	0.99
Waist(cm)	96.0 (79.0-119.0)	95.0 (82.0-121.0)	0.39	-	90.0 (83.0-118.0)	95.0 (79.0-122.0)	0.68	-
Percentile Waist	97.7 (91.9-99.1)	97.1 (91.9-100.0)	0.53	-	97.3 (95.3-98.1)	97.3 (90.7-100.0)	0.48	-
O-SBP (mmHg)	117.5 (102.0-152.3)	121.7 (105.0-163.0)	0.23	-	122.7 (112.7-152.3)	119.0 (102.0-163.0)	0.28	-
Percentile O-SBP	77.7 (47.5-100.0)	92.1 (48.3-100.0)	0.16	-	94.0 (68.8-100.0)	84.5 (45.4-100.0)	0.35	-
O-DBP (mmHg)	67.3 (56.7-82.7)	67.0 (52.0-88.3)	0.89	-	70.7 (62.0-82.3)	68.0 (56.7-88.3)	0.92	-
Percentile O-DBP	66.1 (19.1-93.5)	64.8 (6.6-98.0)	0.67	-	75.1 (40.7-87.1)	66.6 (19.1-98.0)	0.91	-
24h-SBP (mmHg)	116.0 (107.0-130.0)	116.0 (100.0-136.0)	0.73	-	121.0 (118.0-128.0)	115.0 (102.0-136.0)	**0.02**	**0.04**
Percentile 24h-SBP	71.0 (24.5-99.6)	62.4 (4.6-99.7)	0.98	-	92.9 (27.0-99.1)	65.1 (13.3-99.7)	0.30	-
24h-DBP (mmHg)	67.0 (58.0-79.0)	64.0 (55.0-78.0)	0.31	-	68.0 (58.0-78.0)	66.0 (55.0-79.0)	0.54	-
Percentile 24h-DBP	48.6 (4.4-99.0)	34.5 (2.1-98.4)	0.40	-	60.5 (3.7-98.4)	42.5 (2.1-99.0)	0.70	-
TC (mg/dL)	153.0 (105.0-242.0)	169.5 (125.0-216.0)	**0.05**	0.31	165.0 (124.0-242.0)	155.0 (93.0-216.0)	0.66	-
HDL (mg/dL)	49.0 (30.0-81.0)	50.4 (32.0-73.0)	0.29	-	33.0 (30.0-39.0)	50.0 (34.0-81.0)	**0.002***	**0.02**
TG (mg/dL)	75.0 (28.0-285.0)	81.5 (33.0-201.0)	0.94	-	196.0 (126.0-208.0)	72.0 (28.0-168.0)	**0.001***	**0.04**
AST (U/L)	28.0 (15.0-74.0)	23.0 (16.0-33.0)	**0.04**	0.41	31.0 (22.0-59.0)	24.0 (15.0-58.0)	0.25	-
ALT (U/L)	28.0 (13.0-189.0)	20.0 (13.0-37.0)	**0.001***	0.63	34.0 (20.0-157.0)	22.0 (13.0-62.0)	0.21	-
GGT (U/L)	16.0 (9.0-79.0)	13.0 (4.0-23.0)	**0.01**	0.41	16.0 (13.0-47.0)	14.0 (4.0-32.0)	0.26	-
Fast. GLU (mg/dl)	86.0 (73.0-117.0)	88.0 (70.0-101.0)	0.82	-	93.0 (82.0-101.0)	86.0 (70.0-117.0)	0.26	-
Fast. INS (uU/ml)	19.1 (3.0-62.5)	18.5 (5.3-49.8)	0.33	-	27.9 (19.6-49.8)	17.6 (3.0-62.5)	**0.04**	0.07
HOMA-IR	3.7 (0.0-18.0)	4.1 (1.2-12.4)	0.73	-	6.9 (4.0-12.4)	3.7 (0.0-18.0)	0.10	-
FLI	36.0 (6.30-98.70)	26.7 (8.9-80.6)	0.11	-	39.0 (23.4-98.7)	29.9 (6.3-91.7)	0.25	-

FLI: fatty liver index; HDL: HDL cholesterol; O-SBP: Office systolic blood pressure; O-DBP: Office diastolic blood pressure; TC: total cholesterol; TG: Triglycerides; Waist: Waist Circumference.

[1]Wilcoxon-Mann-Whitney U Test; [2] For steatosis, multivariate model includes variables significantly associated with steatosis at univariate analysis plus age, sex and percentile BMI. Due to the low number of patients with MetS (N=5, all males), multivariate models were performed for each characteristic associated with MetS at univariate analysis with age and percentile BMI as covariates * $p<0.05$ after False-Discovery-Rate adjustment for multiple testing

Supplemental Table S3. Fatty Acids Composition of erythrocytes plasma membrane of the obese children divided according to the presence of liver steatosis and metabolic syndrome at baseline

Variable	Steatosis (n=34) Median (range)	Not Steatosis (n=29) Median (range)	p-value[1]	MetS (n= 5) Median (range)	Not MetS (n=53) Median (range)	p-value[1]	Multiv. p-value[2]
PA (%)	22.0 (20.4-24.7)	22.4 (20.7-23.9)	0.50	23.8 (21.3-24.7)	21.7 (20.3-23.7)	**0.0003***	**0.008**
LA (%)	11.7 (7.6-16.2)	11.9 (8.8-17.1)	0.65	11.5 (8.9-12.5)	11.9 (7.6-17.1)	0.21	-
GLA (%)	0.1 (0.0-0.2)	0.1 (0.1-0.3)	0.32	0.1 (0.1-0.2)	0.1 (0.0-0.3)	**0.02**	0.18
DGLA (%)	2.1 (1.6-2.8)	1.8 (1.3-3.2)	0.15	2.1 (1.9-2.8)	1.9 (1.5-3.2)	0.15	-
AA (%)	16.1 (13.1-18.8)	16.5 (13.4-18.9)	0.38	14.4 (13.9-15.2)	16.4 (13.1-18.9)	**0.003***	**0.01**
ALA (%)	0.1 (0.0-0.2)	0.1 (0.0-0.2)	0.95	0.1 (0.1-0.2)	0.1 (0.0-0.2)	0.23	-
EPA (%)	0.4 (0.2-0.7)	0.4 (0.2-0.8)	0.80	0.4 (0.3-0.6)	0.4 (0.2-0.7)	0.21	-
DHA (%)	4.4 (3.2-5.9)	4.3 (2.6-6.1)	0.69	4.5 (3.8-5.8)	4.3 (2.6-5.9)	0.20	-
Omega-3 Index (%)	4.6 (3.5-6.5)	4.6 (2.9-6.6)	0.68	4.9 (4.3-6.5)	4.6 (2.9-6.3)	0.17	-
Omega-3 PUFA (%)	6.3 (5.4-9.0)	6.6 (4.3-8.7)	0.46	6.8 (5.6-9)	6.3 (4.3-8.5)	0.22	-
Omega-6 PUFA (%)	35.2 (31.0-38.7)	35.6 (32.1-39.3)	0.53	33.8 (31.0-35.1)	35.6 (32.6-39.3)	**0.0002***	**0.006**
SFA (%)	40.0 (37.7-42.2)	40.1 (38.6-41.8)	0.74	41.4 (40.9-42.2)	39.8 (37.7-41.6)	**0.004***	0.08
D6D	0.008 (0.004-0.02)	0.009 (0.004-0.02)	0.22	0.01 (0.01-0.02)	0.008 (0.004-0.02)	**0.01**	**0.04**
D5D	7.3 (5.3-12.1)	8.6 (4.6-13.6)	0.13	7.3 (5.1-7.5)	8.2 (4.6-12.8)	0.08	-

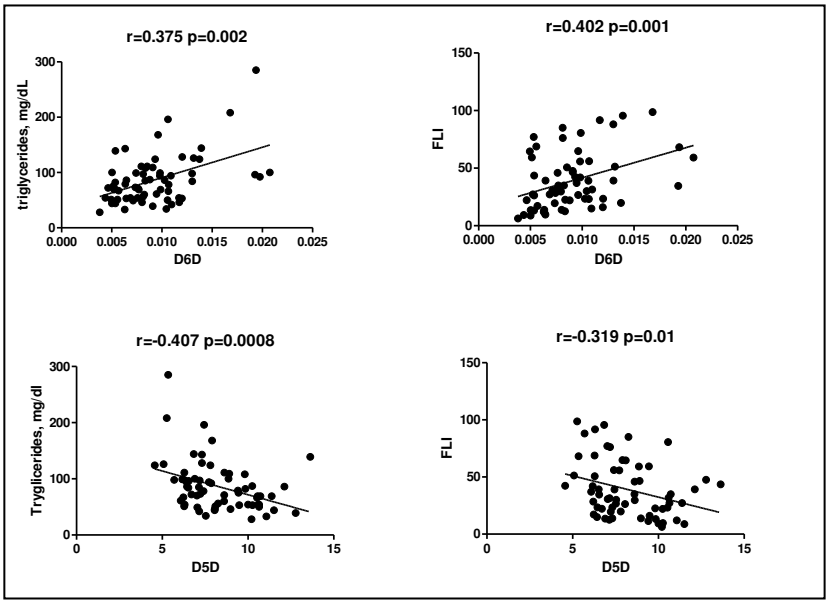
AA: Arachidonic acid; ALA: alpha-Linolenic.PA: palmitic acid; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; D5D: Δ^5-desaturase = C20:4n-6/C20:3n-6; D6D: Δ^6-desaturase = C18:3n-6/C18:2n-6; GLA: gamma-Linolenic acid; Omega-3 Index: sum of Omega 3 fatty acids (EPA and DHA); LA: Linoleic acid; saturated FA (SFA) are calcuated as the sum of C14:0, Palmitic acid, Stearinic acid and Lignocerinic acid; Omega-3 FA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and (DHA); Omega-6 FA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6

[1] T-test or Wilcoxon-Mann-Whitney U Test, as appropriate

[2] Multivariate models were performed for each characteristic associated with MetS at univariate analysis with age and percentile BMI as covariates

* $p<0.05$ after False-Discovery-Rate adjustment for multiple testing

Supplemental Figure S1. Correlations between desaturase activity and features of MetS



D6D: Δ^6-desaturase = C18:3n-6/C18:2n-6; D5D: Δ^5-desaturase = C20:4n-6/C20:3n-6; FLI: Fatty Liver Index.

Study 2: Possible role of omega-3 and omega-6 PUFA and their metabolites via CYP450 in the modulation of blood pressure and arterial stiffness in a sample of obese children

Introduction Study 2

The prevalence of overweight and obesity in children and adolescents has been increasing in the last few decades worldwide. Childhood obesity can affect nearly every organ system and have serious brief term and long term consequences, including metabolic and hemodynamic complications, such as hypertension, insulin resistance and dyslipidemia[283].

Beside the impact of body mass index (BMI) *per se* on blood pressure (BP), several mechanisms are implicated in hemodynamic controls. Dietary habits and in particular lipid intake can influence not only the weight but also haemodynamics[230]. Indeed, in the last years many studies tried to assess the role of several polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA), an omega-6 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which belong to omega-3 PUFA family, suggesting a beneficial cardiovascular effect of omega-3 (n-3) PUFA[68,77], whereas it is not clear whether omega-6 PUFA are cardioprotective or not[188,189].

Omega-6 and omega-3 PUFA can be metabolized via cytochrome P-450 (CYP450) in lipid mediators, which can have profound effects on blood pressure control[230].

AA can be metabolized by different CYP450 enzymes leading to the formation of several compounds: 5-6, 8-9, 11-12, 14-15-epoxyeicosatrienoic acids (EETs) via epoxygenase and 20-hydroxyeicosatetraenoic acid (20-HETE) via hydroxylases. In general, EETs have been shown to play a protective role in cardiovascular diseases through their anti-inflammatory, vasodilating and sodium excreting effects[201,202]. They are further metabolized by soluble epoxide hydrolase (sEH) to dihydroxyeicosatrienoic acids (DHETs), which are less potent or even inactive compounds. Instead, 20-HETE exerts a natriuretic action at kidney level, but, in the systemic vasculature, it induces vasoconstriction[284,285]. Linoleic acid (LA) is also metabolized by CYP450 epoxygenase to 9,10- and 12,13-epoxyoctadecenoic acids (EpOMEs), which are converted by sEH to 9,10- and 12,13-dihydroxyoctadecenoic acids (DiHOMEs). They have a potential toxic cardiovascular effects, probably mediated by mitochondrial dysfunction, and may affect the cardiac inotropic response, but the evidences are rare and diverging[286,287].

Epoxyeicosatetraenoic acids (EEQs), yielded from EPA, have vasodilating and anti-inflammatory properties and are further metabolized by sEH to diydroxxyeicosatetraenoic acids (DiHETEs)[218,221,288]. Epoxydocosapentaenoic acids (EDPs), derived from DHA via CYP-epoxygense, are potent vasodilators[221]; they are hydroxylaletd by sEH to dihydroxydocosapentaenoic acids (DiHDPAs). EPA and DHA can bind also CYP-hydroxylase producing 20-hydroxyeicosapentaenoic acid (20-HEPE) and

22-Hydroxydocosahexaenoic acid (22-HDoHE) respectively, whose biological activity is still largely unknown.

Thus, the aim of the present study was to explore the effect of the contents of different PUFA in erythrocytes plasma membranes, an index of their dietary intake, and of their metabolites via CYP450/sEH in the modulation of haemodynamic parameters and especially BP in a sample of obese children.

Methods Study 2

Obese children were recruited consequently from October 2012 to September 2014, coming from the "Pediatric Obesity Outpatients Unit" of the University Hospital of Verona and of the "Local Health Unit n. 20" of Verona. Inclusion criteria were: children and adolescents aged 5-18 years old; overweight or obesity (BMI$\geq$85th and 95th percentile for sex and age, respectively). We excluded children with hepatic or renal chronic diseases, malignancies, diabetes mellitus, lipid-lowering therapy, secondary causes of obesity.

STUDY DESIGN

The study was conducted according to a cross-sectional observational design. The study was approved by the Ethical Committee of the University Hospital of Verona (CE n. 2218), and written informed consent was obtained from each participant's parents.

ASSESSMENTS

Each child was evaluated in a single occasion, between 8 and 9 a.m. A questionnaire was administered to the patients and to the parents, dealing with medical history, family history, physiological and pathological information and use of drugs. Then, the participants underwent a physical examination. They were advised not to engage in strenuous exercise and to avoid consuming caffeine containing beverages within 12 hours preceding the vascular studies.

During the visit, blood pressure was measured with a semiautomatic oscillometric device (TM-2551, A&D instruments Ltd, Abingdon Oxford, UK) 3 times, 3 minutes apart with the patient lying supine for at least 10 minutes before the first measurement in a room with controlled temperature (22-24°C). The mean value of the 3 clinostatic measurements were calculated and considered for z-score and percentile calculation. Afterward, BP levels were confirmed by a measurement in the sitting position by the oscillometric device and by auscultatory method. Ambulatory blood pressure measurement was recorded with an oscillometric device (Specelabs 90217; Spacelabs Inc., Issaquah, Whashington, USA), which measured BP every 15 minutes during the day and ever 30 minutes during the night. Children and parents recorded physical activities, resting and sleeping time and symptoms on a dedicated diary. After recording, the daytime and nighttime periods (set to default at 0700 and 2200 h, respectively) were adapted to "real" awake and sleep times according to what was declared in the diary of activity.

All of the values derived from BP measurements were transformed in z-score and percentile, according to normative values[257,258]. The 95th of office and ambulatory BP measurements was used as cut-off for hypertension, according to current European guidelines[26].
Body weight, height, and waist and hip circumferences were measured with the patient wearing light clothes. Body weight was measured by a calibrated balance and height by a calibrated stadiometer.
Body mass index (BMI) calculated as weight in Kg divided by the square of height in m; waist/hip ratio was calculated as waist circumference in cm divided by hip circumference in cm and waist/height ratio (WHtR) was calculated as waist circumference in cm divided by height in cm.
Waist circumference was transformed in z-score and percentile according to normative values[260]. Overweight or obesity were defined for BMI≥90th and 95th percentile for sex and age, respectively[255]. WHO reference for BMI was used for categorizing children into the overweight and obese groups[2].
Carotid Intima-media Thickness (cIMT) was assessed by ultrasound of carotid arteries (LogiQ P5 Pro) and the cIMT was estimated tracking the artery wall in the last centimeter of the common carotid artery and calculated by a dedicated software (Multimedia Video Engine II (MVE2) DSP Lab., Pisa CNR, Italy). The relative z-score and percentile were calculated according to reference values[289].
Endothelial function was assessed by ultrasound of the brachial artery using the Flow Mediated Dilatation (FMD) technique according to international guidelines and with the aid of a dedicated hardware (Multimedia Video Engine II (MVE2) DSP Lab., Pisa CNR, Italy)[290]. *Common carotid artery distensibility* (DC) was calculated as: $DC = \Delta A / (A^* \Delta P)$ where A is the diastolic lumen area, ΔA is the stroke change in lumen area, and ΔP is pulse pressure (PP). Changes in diameters were detected using ultrasound B-mode image sequences of the right and left common carotid arteries acquired at different steps and analyzed by the above mentioned automatic system[291]. The relative z-score and percentile were calculated according to reference values[289].

Stiffness Index (SI) and Reflection Index (RI) were estimated by the Digital Volume Pulse (DVP) method and were obtained with the digital photoplethysmography PulseTracePT1000 (MicroMedical Ltd, Gillingham, Kent, UK)[292].

CYP-derived eicosanoids measurement

Plasma samples (500 microliters) were subjected to alkaline hydrolysis and subsequent solid phase extraction was performed as described previously[293]. 500 µL Methanol, 300 µL 10 molar sodium hydroxide and deuterated internal standards were added to 500 µL Plasma. The samples were hydrolyzed for 30 minutes at 60 °C. The solution containing free fatty acids and metabolites were neutralized with acetic acid and adjusted to pH = 6.2.

A solid phase extraction procedure using Agilent Bond-Elut-Certify II was performed as formerly described by Rivera[293].

The LC-ESI-MS/MS method for determination of metabolites was described in detail in previous papers[294].

Red blood cell membrane fatty acids measurement

EDTA-blood tubes were centrifuged, plasma and buffy coat taken off, and erythrocytes frozen at -80C until analysis. Erythrocyte fatty acid composition was analyzed using the HS-Omega-3 Index® methodology as previously described[264,295]. Fatty acid methyl esters were generated from erythrocytes by acid transesterification and analyzed by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu,Duisburg, Germany) equipped with a SP2560, 100-m column (Supelco,Bellefonte, PA) using hydrogen as carrier gas. Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of erythrocytes. A total of 26 fatty acids were identified and quantified.

Results are given as percentage of total identified fatty acids after response factor correction. The coefficient of variation for EPA plus DHA and for most other fatty acids was 4%. Analyzes were quality-controlled according to DIN ISO 15189.

STATISTICS

Data are presented as the median and range unless otherwise stated. The statistical analysis was performed using the software Statistical Package for Social Sciences software (SPSS / PC for Windows version 21.0). Bivariate nonparametric correlations were estimated by Spearman coefficient (r_S).

Differences in the measured parameters between normotensive and hypertensive children were analyzed by nonparametric (Wilcoxon-Mann-Withney U) tests. A two-tailed test with a $p<0.05$ was considered statistically significant.

Results Study 2

General characteristics

We enrolled 70 children from October 2012 to October 2014, aged 5 to 17 years old. Four children had a BMI>90th and 66 children had a BMI >95th percentile for sex and age. All children had a central distribution of adiposity (percentile of waist circumference > 90th percentile). ABPM was available for 68 subjects. Plasma eicosanoids were measured in 69 children. Girls showed higher BP at the ABPM compared to boys. The mean Omega-3 Index was 4.7%. General characteristics of the obese children split in males and females and normotensive and hypertenive, diagnosed on the basis of ABPM (daytime, nighttime or 24-hours SBP or DBP >95th percentile), are detailed in **Table 1**, plasma eicosanoids in **Table 2**. The characteristics of the children divided according to pubertal status are listed in **Supplemental Table S1**.

When dividing the population in two groups, hypertensive (n: 18) and normotensive (n: 50), no differences in FA or their derived-eicosanoids via CYP450/sEH were found between the two groups, except higher concentration of EEQs and DiHETEs in hypertensive compared to normotensive (see Table 2).

Table 1. General characteristics of the obese children divided according to gender and hypertensive status

	Males (n: 40)	Females (n: 30)		Normotensive (n: 50)	Hypertensive (n: 18)	
Variable	**median (range)**	**median (range)**	**p-value***	**median (range)**	**median (range)**	**p-value***
BMI, Kg/m2	28.53 (23.06-42.75)	29.31(24.48-40.65)	0.469	28.33 (23.93;42.75)	30.33 (23.06;38.43)	0.104
z-score BMI	2.13 (1.51-2.83)	2.22 (1.29-3.29)	0.809	2.05 (1.29;2.83)	2.41 (1.75;3.29)	**0.005**
waist circ, cm	96 (79-122)	95.5 (82-119)	0.917	95.5 (80;121)	97.50 (79;122)	0.303
z-score waist circ	1.89 (0-2.4)	2.09 (1.32-3.5)	0.473	1.88 (1.32;2.49)	2.12 (1.65;3.50)	**0.006**
cIMT, mm	0.47 (0.33-0.61)	0.42 (0.32-0.55)	**0.030**	0.44 (0.32;0.61)	0.46 (0.32;0.56)	0.330
z-score cIMT	2.15 (-1.86-3.81)	1 (-2.22-4.95)	0.091	1.20 (-2.22;3.67)	1.78 (-1.86;4.95)	0.334
DC, 10-3/KPa	39.68 (19.46-54.01)	43.81 (27.42-63.1)	**0.032**	42.67 (19.46;63.10)	37.59 (19.71;58.61)	0.104
z score DC media	-1.52 (-4.17-0.01)	-1.12 (-2.85-0.47)	0.102	-1.15 (-4.17;0.47)	-1.65 (-4.12;-0.11)	0.080
CC, mm2/KPa	1.16 (0.59-1.51)	1.16 (0.65-1.79)	0.907	1.20 (0.77;1.68)	1.04 (0.59;1.79)	0.072
FMD, %	7.6 (-1.1-16)	6.2 (-0.3-14.2)	1.00	7 (-1.10;14.20)	6.90 (-0.30;16)	0.670
RI, %	60.33 (33-83.33)	57.83 (20.33-81.67)	0.520	59.33 (31;81.67)	57.67 (20.33;83.33)	0.693
SI, m/s	6.19 (5.03-9.07)	6.1 (4.73-8.44)	0.887	6.21 (5.03;8.44)	6.05 (4.73;9.07)	0.822
O-SBP, mmHg	119 (102-163)	115 (105-143)	0.469	117 (102;163)	122.50 (109;160.33)	**0.026**
z-score O-SBP	1.04 (-0.13-5.21)	0.78 (-0.12-3.21)	0.469	0.75 (-0.13;5.21)	1.65 (-0.04;4.95)	**0.007**
O-DBP, mmHg	68.33 (52-88.33)	66.5 (56.67-83.67)	0.227	66.50 (52;88.33)	74.83 (58;83.33)	0.162
z-score O-DBP	0.4 (-1.51-2.06)	0.4 (-0.74-1.78)	0.809	0.36 (-1.51;2.06)	0.89 (-0.62;1.72)	**0.023**
24h-SBP, mmHg	118 (107-136)	112 (100-130)	**0.004**	114 (100;125)	121 (110;136)	**≤0.001**
z-score 24h-SBP	0.64 (-0.63-2.77)	0.04 (-1.69-2.66)	0.141	0.04 (-1.69;1.70)	1.30 (0.05;2.77)	**≤0.001**
24h-DBP, mmHg	68 (58-79)	64 (55-74)	**0.006**	65 (57;79)	69 (55;79)	**0.001**
z -score 24h-DBP	0.17 (-1.79-2.32)	-0.4 (-2.02-1.63)	**0.050**	-0.39 (-1.79;2.32)	0.41 (-2.02;2.14)	**0.001**
Day-SBP, mmHg	123 (109-142)	115 (102-137)	**0.014**	117.50 (102;132)	126 (113;142)	**≤0.001**
z-score day-SBP	0.58 (-1.1-2.93)	0.19 (-1.84-2.65)	0.327	0.20 (-1.84;1.35)	1.26 (0.01;2.93)	**≤0.001**
Day-DBP, mmHg	71 (63-85)	67 (58-80)	0.055	68.50 (59;79)	73.50 (58;85)	**0.013**
z-score day-DBP	-0.2 (-1.57-2.23)	-0.85 (-2.07-1.24)	**0.025**	-0.54 (-1.90;1.15)	0.21 (-2.07;2.23)	**≤0.001**
Night-SBP, mmHg	108.5 (100-124)	104.5 (92-128)	0.592	105.50 (92;117)	116 (106;128)	**≤0.001**
z-score night-SBP	0.8 (-0.28-2.63)	0.68 (-0.71-3.09)	0.801	0.51 (-0.71;1.59)	1.86 (-0.15;3.09)	**≤0.001**
Night-DBP, mmHg	60.5 (49-73)	57 (48-69)	**0.011**	57 (48;66)	64 (52;73)	**≤0.001**
z-score night-DBP	0.73 (-1.35-2.56)	0.36 (-1.18-1.7)	0.078	0.30 (-1.35;1.58)	1.25 (-0.48;2.56)	**≤0.001**

BMI: body mass index; waist circ: waist circumference; cIMT: carotid intima-media thickness; DC: carotid distensibility; CC: carotid compliance; FMD: flow-mediated dilation; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.
*Wilcoxon-Mann-Whitney U test.

Table 2. Plasma eicosanoids and erythrocyte membrane FA profiles in obese children, divided according to gender and hypertensive status

	Male (n: 40)	Female (n: 30)		Normotensive (n: 50)	Hypertensive (n: 18)	
Variable	**Median (Range)**	**Median (Range)**	**p-value***	**Median (Range)**	**Median (Range)**	**p-value***
EpOMEs, ng/mL	11.56 (3.98;24.94)	10.41 (5.82;19.12)	0.561	10.49 (3.98;23.40)	10.75 (4.91;24.94)	0.687
DiHOMEs, ng/mL	7.19 (2.28;44.93)	6.33 (2.72;13.55)	0.280	6.33 (2.28;24.73)	7.46 (3.11;44.93)	0.310
EETs, ng/mL	7.5 (4.55;13.51)	7.64 (4.57;13.62)	0.835	7.33 (4.55;13.62)	8.37 (4,59;10.46)	0.108
DHETs, ng/mL	4.14 (2.18;6.84)	4.03 (2.79;5.59)	0.840	3.99 (2.29;5.59)	4.03 (2.18;6.52)	0.334
EEQs, ng/mL	0.34 (0.18;1.22)	0.37 (0.15;1.64)	0.593	0.33 (0.15;1.64)	0.41 (0.18;0.99)	**0.038**
DiHETEs, ng/mL	0.92 (0.57;3.55)	0.91 (0.45;2.04)	0.393	0.85 (0.45;3.55)	1.16 (0.56;2.97)	**0.017**
EDPs, ng/mL	2.86 (1.79;7.01)	3.12 (1.99;6.86)	0.208	2.85 (1.84;7.01)	3.31 (1.79;5.35)	0.190
DiHDPAs, ng/mL	0.9 (0.58;1.83)	0.92 (0.62;1.46)	0.581	0.88 (0.58;1.83)	0.91 (0.64;1.46)	0.604
LA, %	11.7 (7.64;16.17)	11.93 (8.81;17.14)	0.627	11.68 (7.64;16.17)	12.35 (10.07;17.14)	0.116
GLA, %	0.11 (0.04;0.29)	0.09 (0.05;0.33)	0.326	0.10 (0.04;0.15)	0.11 (0.06;0.33)	0.422
DGLA, %	1.99 (1.57;3.2)	2 (1.27;2.73)	0.836	1.89 (1.27;2.78)	1.99 (1.57;3.20)	0.511
AA, %	15.96 (13.06;18.73)	16.38 (13.14;18.91)	0.093	16.38 (13.06;18.91)	15.58 (13.36;18.73)	0.087
ALA, %	0.08 (0.02;0.19)	0.08 (0.02;0.23)	0.533	0.07 (0.02;0.23)	0.08 (0.03;0.19)	0.966
EPA, %	0.37 (0.22;0.74)	0.4 (0.19;0.81)	0.391	0.37 (0.19;0.74)	0.41 (0.25;0.81)	0.149
DHA, %	4.3 (2.58;6.13)	4.35 (2.81;5.96)	0.437	4.36 (2.81;6.13	4.27 (2.58;5.96)	0.497
Omega-3 Index, %	4.63 (2.87;6.61)	4.62 (3.00;6.57)	0.440	4.62 (3.00;6.61)	4.60 (2.87;6.57)	0.761
Omega-3 PUFA, %	6.33 (4.32;9.02)	6.3 (4.46;8.7)	0.444	6.43 (4.51;9.02)	6.26 (4.32;8.70)	0.616
Omega-6 PUFA, %	35.36 (31.02;38.68)	35.55 (33.04;39.29)	0.211	35.6 (31.02;39.29)	35.25 (32.07;38.68)	0.611
SFA, %	40.45 (38;42.15)	39.65 (37.65;41.61)	**0.044**	40.18 (37.65;42.07)	39.60 (38.00;42.15)	0.404
D5	7.62 (4.56;11.38)	8.06 (5.70;13.63)	0.527	8.06 (5.08;13.63)	7.35 (4.56;10.64)	0.373
D6	0.008 (0.001;0.002)	0.008 (0.001;0.002)	0.301	0.01 (0.004;0.002)	0.01 (0.004;0.002)	0.552

DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; AA: Arachidonic acid; ALA: alpha-Linolenic.PA: palmitic acid; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; Omega-3 Index: (EPA + DHA)/total FA*100; LA: Linoleic acid; saturated FA (SFA) are calcuated as the sum of C14:0, Palmitic acid, Stearinic acid and Lignocerinic acid; Ω-3 FA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and DHA; Ω-6 FA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6.

* Wilcoxon-Mann-Whitney U test.

Correlations between fatty acids and the relative CYP450/sEH metabolites

LA was directly correlated with DiHOMEs in the whole population, with 12,13-EpOME and DiHOMEs in hypertensive and with the single isomer 9,19-DiHOME in normotensive children.

EPA directly correlated with EEQs and DiHETEs in the whole population and in the subgroups, with stronger correlations in hypertensive subjects. DHA was directly correlated with EDPs and DiHDPAs in the whole population and in normotensive and with EDPs in hypertensives. No correlations where found between AA and the relative metabolites via CYP450/sEH. (**Table 3a, 3b, 3c, 3d**)

Table 3a. Correlations between LA and the relative eicosanoids via CYP450/sEH

LA			
	Whole population	Normotensive	Hypertensive
9,10-EpOME	-0.102	0.06	0.289
12,13-EpOME	0.094	0.06	**0.315***
9,10-DiHOME	**0.438^**	**0.311***	**0.679^**
12,13-DiHOME	**0.399^**	0.265	**0.618^**

In the table are reported the r_S values of the correlations. * : significance below 0.05; ^: significance below 0.01. Underlined correlations are significant after adjustment for sex, age and BMI. DiHOME: dihydroxyoctadecenoic acid; EpHOME: epoxyoctadecenoic acid; LA: Linoleic Acid.

Table 3b. Correlations between AA and the relative eicosanoids via CYP450/sEH

AA			
	Whole population	Normotensive	Hypertensive
5,6-EET	0.029	0.152	-0.049
8,9-EET	0.061	0.162	-0.064
11,12-EET	0.049	0.096	0.177
14,15-EET	0.040	0.114	0.074
5,6-DHET	0.032	0.196	-0.251
8,9-DHET	0.105	0.201	-0.051
11,12-DHET	0.014	0.166	-0.418
14,15-DHET	0.210	0.218	0.220
20-HETE	-0.057	-0.076	-0.076

In the table are reported the r_S values of the correlations. * : significance below 0.05; ^: significance below 0.01. Underlined correlations are significant after adjustment for sex, age and BMI. AA: Arachidonic Acid; EET: epoxyeicosatrienoic acid; DHET: Dihydroxyeicosatrienoic acids.

Table 3c. Correlations between EPA and the relative eicosanoids via CYP450/sEH

EPA			
	Whole population	Normotensive	Hypertensive
8,9-EEQ	**0.434^**	0.267	**0.784^**
11,12-EEQ	**0.575^**	**0.482^**	**0.794^**
14,15-EEQ	**0.641^**	**0.590^**	**0.808^**
17,18-EEQ	**0.552^**	**0.509^**	**0.643^**
5,6-DiHETE	**0.512^**	**0.441***	**0.652^**
8,9-DiHETE	**0.398^**	**0.337***	0.446
11,12-DiHETE	0.217	0.244	-0.023
14,15-DiHETE	**0.464^**	**0.393^**	**0.532***
17,18-DiHETE	**0.484^**	**0.431^**	**0.440***

In the table are reported the r_S values of the correlations. * : significance below 0.05; ^: significance below 0.01. Underlined correlations are significant after adjustment for sex, age and BMI. EEQ: epoxyeicosatetraenoic acid; DiHETE: Dihydroxyeicosatetraenoic acids; EPA: Eicosapentaenoic acid.

Table 3d. Correlations between DHA and the relative eicosanoids via CYP450/sEH

DHA			
	Whole population	Normotensive	Hypertensive
7,8-EDP	**0.482^**	**0.553^**	**0.666^**
10,11-EDP	**0.375^**	**0.360***	**0.569^**
13,14-EDP	**0.467^**	**0.515^**	**0.563^**
16,17-EDP	**0.502^**	**0.591^**	**0.546^**
19,20-EDP	**0.456^**	**0.581^**	0.473
7,8-DiHDPA	**0.453^**	**0.521^**	0.324
10,11-DiHDPA	0.220	**0.310***	0.205
13,14-DiHDPA	**0.256***	**0.325***	0.290
16,17-DiHDPA	**0.386^**	**0.531^**	0.086
19,20-DiHDPA	**0.383^**	**0.565^**	0.010
22-HDHA	0.039	0.067	-0.015

In the table are reported the r_S values of the correlations. * : significance below 0.05; ^: significance below 0.01. Underlined correlations are significant after adjustment for sex, age and BMI. EDP: epoxydocosapentaenoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DHA: Docosahexaenoic acid.

Correlations of CYP450/sEH – derived eicosanoids with blood pressure and vascular tests

In the whole population, total DiHOME and 9,10- DiHOME correlated with office and nighttime DBP and the relative z-score. Both isomer and total DiHOME were also directly correlated to RI. 20-HETE was directly correlated with office DBP and its z-score. (The correlations of eicosanoids with blood pressure, z-score of blood pressure and vascular tests are detailed in **Table 4** and **Supplemental Tables S2** and **S3**, respectively)

Correlations of erythrocyte membrane FA with BP and vascular tests

RI was directly correlated with LA and inversely with DHA and total omega-3 PUFA. The z-score of IMT was inversely correlated with EPA. (The correlations of fatty acids with blood pressure, z-score of blood pressure and vascular tests are detailed in **Table 5, Supplemental Table S4 and Table 6** respectively)

Correlations of blood pressure with vascular tests

DC and the relative z-score were inversely correlated with almost all office and ambulatory BP measurements. IMT directly correlated with 24-hours SBP and nighttime SBP. RI showed an inverse correlation with office SBP. (The correlations of blood pressure with vascular tests are detailed in **Table 7** and correlations of z-score of blood pressure with vascular tests are detailed in **Supplemental Table S5**)

Table 4. Correlation between eicosanoids and blood pressure in the total population

Whole Population								
	Office SBP	**Office DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night-SBP**	**Night-DBP**
EpOMEs	-0.044	0.121	0.009	0.097	-0.010	0.098	-0.050	0.021
DiHOMEs	-0.076	**0.288*** 9.10-DiHOME **0.323^**	0.067	0.179	-0.015	0.082	0.141	**0.286*** 9.10-DiHOME **0.290***
EETs	0.082	0.046	0.056	0.070	0.042	0.069	0.053	0.003
DHETs	0.140	0.084	0.059	0.029	0.099	0.072	0.035	0.009
EEQs	-0.084	-0.147 8.9-EEQ **-0.264***	0.083	0.066	0.074	0.052	0.090	-0.025
DiHETEs	-0.057	-0.119	0.153	0.149	0.164	0.088	0.119	0.154
EDPs	-0.078	-0.110	0.021	-0.043	0.033	-0.038	-0.040	-0.093
DiHDPAs	-0.022	-0.043	-0.010	-0.067	0.045	-0.042	-0.136	-0.106
22-HDHA	-0.055	-0.048	-0.087	-0.181	-0.101	-0.173	-0.065	-0.201
20-HETE	0.121	**0.265***	-0.006	0.059	-0.102	-0.037	0.104	0.178

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI. DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Table 5. Correlation between erythrocyte membrane fatty acids and blood pressure measurements in the whole population, and after division in hypertensive and normotensive children.

Whole population								
	Office SBP	**Office DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night SBP**	**Night DBP**
Omega-6 PUFA	-0.04	0.12	-0.22	-0.02	**-0.247***	0.01	-0.15	-0.09
LA	-0.11	0.14	0.1	0.17	0.07	0.14	0.06	0.14
AA	-0.06	-0.14	**-0.313***	-0.21	**-0.267***	-0.11	**-0.295***	**-0.344^**
DGLA	0.08	0.13	0.15	0.13	0.1	0.07	0.14	0.12
Omega-3 PUFA	0.1	-0.13	0.05	-0.11	0.08	-0.09	0.06	-0.03
ALA	-0.11	0.08	-0.07	0.05	-0.07	0.06	-0.01	0.01
EPA	0.04	-0.15	0.15	-0.07	0.17	-0.08	0.15	0.01
DHA	0.1	-0.11	0.02	-0.14	0.04	-0.13	0.01	-0.04
Hypertensive								
	Office SBP	**Office DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night-SBP**	**Night-DBP**
Omega-6 PUFA	0.319	0.270	-0.101	-0.244	-0.280	-0.255	0.064	-0.162
LA	-0.006	0.221	-0.359	-0.244	-0.384	-0.263	-0.383	-0.246
AA	0.046	-0.145	0.101	-0.238	-0.031	-0.136	0.253	-0.245
DGLA	0.096	0.184	0.351	0.030	0.239	-0.117	0.137	0.144
Omega-3 PUFA	-0.097	-0.448	0.229	-0.081	0.234	-0.018	0.242	-0.102
ALA	-0.194	-0.037	-0.158	0.137	-0.102	-0.082	0.07	0.179
EPA	**-0.608***	**-0.563^**	-0.067	-0.244	0.049	-0.172	-0.178	-0.383
DHA	-0.070	-0.395	0.152	-0.051	0.184	-0.002	0.128	-0.063
Normotensive								
	Office SBP	**Office DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night SBP**	**Night DBP**
Omega-6 PUFA	-0.58	0.042	-0.245	0.066	-0.229	0.113	-0.188	0.009
LA	-0.180	0.089	0.056	0.233	0.062	0.208	0.052	0.229
AA	-0.034	-0.144	**-0.325***	-0.221	-0.268	-0.124	**-0.335***	**-0.340***
DGLA	0.040	0.071	0.078	0.103	0.035	-0.084	0.117	0.044
Omega-3 PUFA	0.183	0.006	0.052	-0.126	0.085	-0.134	0.062	-0.013
ALA	-0.151	0.180	-0.123	0.102	-0.093	0.101	-0.084	-0.030
EPA	0.169	-0.049	0.060	-0.131	0.092	-0.136	0.090	-0.044
DHA	0.188	0.025	0.057	-0.151	0.067	-0.176	0.025	-0.025

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. AA: Arachidonic acid; ALA: alpha-Linolenic; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LA: Linoleic acid; Omega-3 PUFA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and (DHA); Omega-6 PUFA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6

Table 6. Correlations between erythrocyte membrane fatty acids and vascular tests in the whole population, and after division in hypertensive and normotensive children.

	cIMT	Z-score IMT	DC	Z-score DC	RI	SI	FMD %
Whole population							
Omega-6 PUFA	-0.058	0.004	0.050	-0.027	0.142	-0.011	-0.209
LA	0.086	0.105	0.131	0.106	**0.354^**	0.147	-0.130
AA	-0.176	-0.144	0.097	0.051	-0.227	-0.222	-0.011
DGLA	-0.046	-0.030	-0.191	-0.207	0.023	0.123	0.211
Omega-3 PUFA	-0.129	-0.236	-0.162	-0.105	**-0.291***	-0.222	0.198
ALA	0.064	0.059	0.022	0.016	0.008	-0.009	-0.031
EPA	-0.192	**-0.272***	-0.067	-0.012	-0.145	-0.175	0.164
DHA	-0.102	-0.200	-0.106	-0.054	**-0.284***	-0.197	0.133
Hypertensive							
Omega-6 PUFA	-0.070	-0.040	-0.191	-0.188	0.243	-0.017	-0.347
LA	-0.080	-0.003	0.197	0.197	0.468	0.412	-0.382
AA	-0.267	-0.269	0.038	0.030	-0.199	**-0.571***	-0.132
DGLA	0.130	0.110	-0.235	-0.259	0.113	0.284	0.421
Omega-3PU FA	-0.063	-0.131	-0.165	-0.156	-0.439	**-0.527***	0.001
ALA	0.375	0.304	-0.121	-0.062	-0.027	0.155	0.084
EPA	-0.246	-0.308	0.403	0.393	-0.290	-0.189	0.271
DHA	-0.028	-0.057	-0.085	-0.088	-0.419	**-0.519***	-0.144
Normotensive							
Omega-6 PUFA	-0.001	0.111	0.113	0.017	0.073	-0.041	-0.122
LA	0.142	0.181	0.132	0.108	0.289	-0.002	-0.185
AA	-0.067	-0.016	0.088	0.043	-0.287	-0.141	0.166
DGLA	-0.144	-0.132	-0.179	-0.202	-0.005	0.075	0.084
Omega-3 PUFA	-0.130	-0.250	-0.163	-0.087	-0.224	-0.053	0.277
ALA	-0.085	-0.091	0.081	0.043	0.061	-0.092	-0.106
EPA	-0.243	**-0.359***	-0.174	-0.099	-0.126	-0.130	0.142
DHA	-0.097	-0.217	-0.137	-0.061	-0.202	0.005	0.258

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The underlined values represent the correlations that remain significant after adjustment for sex, age and BMI. AA: Arachidonic acid; ALA: alpha-Linolenic; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LA: Linoleic acid; Omega-3 PUFA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and (DHA); Omega-6 PUFA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6; cIMT: carotid intima-media thickness; DC: carotid distensibility; FMD: flow-mediated dilation; RI: reflextion index; SI: stiffness index.

Table 7. Correlations between blood pressure and vascular test in the whole population, and after division in hypertensive and normotensive children.

	O-SBP	**O-DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night-SBP**	**Night-DBP**
Whole Population								
cIMT	0.23	0.001	0.27	0.12	0.19	-0.03	0.33	0.21
z-Score cIMT	0.15	-0.01	0.22	0.16	0.14	0.02	**0.293***	00.22.00
DC medio	**-0.627^**	**-0.332^**	**-0.449^**	**-0.272***	**-0.397^**	-0.17	**-0.490^**	**-0.336^**
z-Score DC	**-0.572^**	**-0.332^**	**-0.395^**	**-0.285***	**-0.329^**	-0.19	**-0.464^**	**-0.330***
FMD	-0.02	-0.03	0.06	0.11	0.08	0.12	0.01	0.11
RI	-0.255*	-0.02	-0.1	0.01	-0.12	-0.04	-0.07	0.07
SI	-0.05	0.22	-0.15	0.12	-0.14	0.1	-0.24	0.03
Hypertensive								
cIMT	0.058	-0.104	-0.038	-0.115	-0.193	-0.264	0.303	0.091
z-Score cIMT	-0.053	-0.130	-0.126	-0.093	-0.272	-0.241	0.197	0.122
DC medio	**-0.744^**	-0.200	-0.457	0.01	**-0.602***	-0.161	**-0.517***	-0.178
z-Score DC	**-0.740^**	-0.169	-0.487	-0.040	**-0.630***	-0.206	-0.479	-0.214
FMD	0.054	0.203	**0.635^**	**0.575***	**0.582***	**0.548***	0.053	0.388
RI	0.021	-0.080	-0.180	0.073	-0.093	0.156	-0.180	0.016
SI	-0.059	0.333	0.137	0.300	0.122	0.419	-0.265	0.245
Normotensive								
cIMT	0.276	-0.004	**0.321***	0.199	0.257	0.051	**0.301***	0.230
z-Score cIMT	0.175	-0.006	**0.291***	0.252	0.232	0.107	0.273	0.258
DC medio	**-0.582^**	**-0.343***	**-0.432^**	-0.254	**-0.374***	-0.149	**-0.471^**	**-0.330***
z-Score DC	**-0.504^**	**-0.356***	**-0.354***	-0.259	-0.282	-0.151	**-0.417^**	**-0.303***
FMD	0.04	-0.092	0.146	0.139	-0.146	-0.113	-0.109	-0.059
RI	**-0.390***	-0.027	-0.155	-0.04	-0.152	-0.098	-0.075	0.103
SI	-0.061	0.145	-0.253	-0.018	-0.257	-0.004	-0.231	-0.082

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The underlined values represent the correlations that remain significant after adjustment for sex, age and BMI. cIMT: carotid intima-media thickness; DC: carotid distensibility; CC: carotid compliance; FMD: flow-mediated dilation; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

In an exploratory analysis, we tested whether the correlations of PUFA and the eicosanoids with BP and vascular tests were different in the two groups, as detailed in the following paragraphs and in supplemental material.

Hypertensive children:

Correlations of CYP450/sEH – derived eicosanoids with blood pressure and vascular tests

EEQs, an in particular two isomers 11,12- and 17,18-EEQ, and DiHETEs, inversely correlated with office SBP. EDPs as well showed inverse correlations in particular with office DBP.

EEQs were also inversely correlated with IMT and directly with DC. (Correlations of eicosanoids with BP values are detailed in **Table 8** and those of the z-score of BP in **Supplemental Table S6**, Correlations of eicosanoids with vascular tests are presented in **Table 9**)

Correlations of erythrocyte membranes FA with blood pressure and vascular tests

In hypertensive children, we found inverse correlations between total omega-6 PUFA with different ABPM, and in particular, LA inversely correlated with BP. AA inversely correlated with SI. Total omega-3 PUFA directly correlated with some BP measurements. (Correlations of FA with BP values are detailed in **Table 5** and those of the z-score of BP in **Supplemental Table S4**. Correlations of vascular tests are presented in **Table 6**)

Correlations of blood pressure with vascular tests

Office SBP and its z-score, 24-hours SBP were inversely correlated with DC and its z-score. Daytime SBP, daytime DBP and its percentile directly correlated with FMD. SI was inversely correlated with z-score of nighttime SBP. (The correlations of blood pressure with vascular tests are detailed in **Table 7** and correlations of z-score of blood pressure with vascular tests are detailed in **Supplemental Table S5**)

Regressions

Most of the correlations remained significant after adjustment for sex, age and BMI, as detailed in the tables depicting the correlations.

Table 8 Correlations between eicosanoids and blood pressure measurements in hypertensive children

Hypertensive								
	Office SBP	**Office DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night-DBP**	**Night-DBP**
EpOMEs	-0.256	-0.094	-0.315	-0.291	-0.428	-0.380	-0.271	0.068
DiHOMEs	-0.104	0.232	-0.252	-0.098	-0.363	-0.123	-0.266	0.037
EETs	-0.108	-0.287	-0.211	0.071	-0.087	0.183	-0.311	0.106
DHETs	0.016	-0.109	-0.034	-0.050	0.112	0.046	-0.102	-0.047
EEQs	**-0.539*** 11.12-EQQ **-0.694*** 17.18-EEQ **-0.584***	-0.299	-0.2761 11.12-EQQ **-0.492***	-0.117	-0.080	-0.038	-0.439 11.12-EQQ **-0.559*** 17.18-EEQ **-0.615^**	-0.216
DiHETEs	-0.437 17.18-DiHETE **-0.578***	-0.120	-0.233 17.18-DiHETE **-0.512***	0.142	-0.076	0.205	-0.348 17.18-DiHETE **-0.596***	0.089
EDPs	-0.176	**-0.519*** 7.8-EDP **-0.523*** 10.11-EDP **-0.540*** 16.17-EDP **-0.509***	-0.093	-0.296	-0.038	-0.229	-0.361 19.20-EDP **-0.598***	-0.240
DiHDPAs	-0.090	-0.382	0.093	-0.183	0.277	-0.070	-0.195	-0.350
22-HDHA	-0.135	-0.191	-0.252	-0.369	-0.107	-0.326	-0.109	**-0.503***
20-HETE	-0.074	-0.147	-0.275	-0.213	-0.297	-0.254	0.033	-0.085

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI. DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Table 9. Correlations between eicosanoids and vascular tests in hypertensive children.

Hypertensive							
	cIMT	**Z-Score cIMT**	**DC**	**Z-Score DC**	**RI**	**SI**	**FMD**
EpOMEs	-0.063	0.034	0.050	0.093	0.206	0.275	-0.050
DiHOMEs	0.067	0.071	-0.132	-0.120	**0.500*** 12.13-DiHOME **0.544***	**0.518*** 9.10-DiHOME **0.518***	-0.004
EETs	-0.231	-0.061	0.314	0.214	0.294	-0.037	0.118
DHETs	-0.301	-0.273	0.155	0.071	0.032	0.066	0.296
EEQs	**-0.605^** 8.9-EEQ **-0.439*** 14.15-EEQ **-0.609^** 17.18-EEQ **-0.615^**	**-0.620*** 8.9-EEQ **-0.539*** 14.15-EEQ **-0.657^** 17.18-EEQ **-0.637***	0.344 11.12-EEQ **0.575***	0.388 11.12-EEQ **0.582***	-0.278	-0.090	0.288
DiHETEs	-0.354 5.6-DiHETE **-0.559*** 17.18-DiHETE **-0.550***	-0.323 5.6-DiHETE **-0.577***	0.290	0.209	0.134	0.153 11.12-DiHETE **0.558***	0.375 5.6-DiHETE **-0.576***
EDPs	-0.098	0.002	0.312	0.237	-0.242	**-0.527*** 13.14-EDP **-0.596***	-0.220
DiHDPAs	-0.114	-0.104	0.148	0.086	-0.071	-0.131	-0.091
22-HDHA	-0.367	-0.461	0.011	0.050	-0.306	0.128	0.054
20-HETE	-0.058	0.034	0.073	0.005	0.346	0.022	0.023

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI. DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; cIMT: carotid intima-media thickness; DC: carotid distensibility; FMD: flow-mediated dilation; RI: reflextion index; SI: stiffness index.

Discussion Study 2

Our main hypothesis was that dietary PUFA could affect hemodynamics, and in particular blood pressure, through the formation of the specific PUFA-derived metabolites via CYP450/sEH. Scarce are the evidence linking together the amount of fatty acids introduced by the diet and their metabolites via CYP450/sEH with hemodynamics in humans and, to our knowledge, this is the first study that investigated this link in children. Apart from the AA-EETs-DHETs cascade of metabolites, we found an interesting correlation between the metabolites via CYP450, supporting the idea that from a dietary assumption of specific omega-3 PUFA (or at least a higher storage in plasma membrane) derives a higher production of their metabolites.

Anyhow, our primary results do not support a major role of EETs/EEQs/EDPs on blood pressure maintenance in obese children, since they neither directly correlate with BP or other vascular exams in the whole sample nor are significantly different in normotensive as compared to hypertensive subjects, except EEQs that resulted higher in hypertensive children. Anyhow only a weak inverse correlation of 8,9-EEQ with office-DBP was detectable in the whole sample. Moreover, we found also a weak inverse association between 20-HETE, a well-known vasoconstrictor compound, and office DBP but not with other BP measurements.

The exploratory analyses in the subgroup of hypertensive children, instead seems to reveal a potential role of some metabolites (especially EEQs and EDPs) on BP. Especially EEQs, and in particular 11,12- and 17,18- isomers, showed inverse associations with several BP measurements and with the markers of vascular structure and function (namely, carotid IMT and distensibility) in hypertensive obese children. Even if this is only an exploratory analysis and should be examined with caution, it is compatible with the hypothesis that endothelium have other dominant vasodilatory substances such a nitric oxide and prostacyclin, so that the effect of endothelial derived hyperpolarizing factors (such as EETs/EEQs/EDPs) is viewable only in circumstances when these main mechanisms have been impaired. Interestingly, we found also a stronger association of FMD, an index of endothelial function, and daytime BP.

A complex link between PUFA and BP is supported also by the literature where the putative beneficial effect of omega-3 PUFA on BP and subsequent cardiovascular events is often blurred, being evident in some trials but not in other or meta-analyses[76,77], with some studies available also in children[111,296][252]. We could hypothesize that also in these studies differences in the examined population could have led to different results.

Despite plenty of studies in animal models[297], especially rats, the evidence that EETs could affect BP in humans is scanty. In particular, our group have found lower plasma EETs in patients affected by renovascular hypertension as compared to essential hypertension and controls[206] and an augmented production of EETs in plasma and placentas obtained by preeclamptic women[207,208].

Little is known about the specific actions of the EPA/DHA-derived metabolites via CYP450/sEH on hemodynamic modulation both in animal models and humans but a few studies support a protective effect of blood pressure, at least for

some single isomers[57,221] suggesting that their effect could be even more potent with respect to EETs.

In our sample, the levels of the EPA-derived metabolites via CYP450/sEH were higher in hypertensive children, compared to normotensive. In the exploratory analysis, we also found that epoxymetabolites of EPA, and the relative diols, are inversely associated to SBP, whereas the epoxydes of DHA were inversely associated to DBP, especially in hypertensive subjects. Interestingly, EEQs and DiHETEs were also inversely associated with carotid IMT and distensibility in hypertensive but not in normotensive obese children. EPA itself showed a trend toward an inverse association with SBP in hypertensive subjects, even if it did not reach the statistically significance.

Interestingly also some other study, according to our observation, suggests that EPA and DHA exert a stronger effect in hypertensive subjects as compared to normotensive [75–77].

The observational design of the study and the exploratory analysis suggest to look at all these associations with caution. Anyhow, we hypothesize that EPA could exert a somehow protective action on blood pressure through their metabolites via CYP450/sEH, probably mediated by a beneficial influence on vascular structure and function, and this effect could be stronger in hypertensive obese children than in normotensive. The stronger correlations of EPA with its metabolites in hypertensive rather than in normotensive subjects support the hypothesis that their effect becomes more important when BP is higher.

Indeed, DiHOME, the diols derived from LA via CYP450 and sEH metabolism, showed a direct correlation with diastolic BP in normotensive but not in hypertensive, as well as a direct association with RI suggesting that they negatively affect vascular stiffness and BP but only in normotensive obese children. Very little is known about the actions of EpOMEs and DiHOMEs; first data indicated a toxic effect of EpOME and probably of DiHOME that could be dose-dependent; they also could affect cardiac contractility, but the results are not always consistent[286,287]. On the other hand, a recent study has proposed a possible protective effect of 12,13-DiHOME on metabolic profile, due to its action on brown adipose tissue uptake of fatty acids[298]. Furthermore, an epoxy-keto derivative of LA has been identified as a possible stimulating factor for aldosetrone secretion, but it is not to date understood its precursor and its metabolic pathway[299]. Furthermore, we found that LA was inversely correlated to BP in hypertensive subjects, suggesting a possible beneficial effect of LA on BP. These data are not easy to explain, considering at the same time the results of the metabolite of LA. Anyhow it should be considered that from each fatty acid derive a range of lipid mediators, which can have different actions. Moreover, also in a previous study in animal models it has been found a discrepancy in the effect of dietary intake of fatty acids, i.e. LA and ALA, on the composition of PUFA and their related metabolites[300].

As above mentioned, the study has limitations: the sample size is relatively low, which can primarily expose to a problem of statistical power, thus, considering also the observational design of the study and the findings from the exploratory

analysis, the results should be carefully evaluated. Anyhow, some interesting correlations were found suggesting a link between the consumption of PUFA, their metabolic pathway via CYP450 and the haemodynamic homeostasis in obese children, opening this field to further investigations even in children. Furthermore, because of the major accuracy, we decided to define hypertension on the basis of ABPM, which is however not considered as the standard method for BP assessment in the clinical setting. Anyhow, we obtained also several office BP measurements, both in the supine position and in the recommended sitting position, that strengthened the results derived from ABPM.
Globally, our data could suggest that single lipid mediators may exert specific actions in hemodynamic control in obese children, that may be different in hypertensive rather than in normotensive children. What remains to understand are the regulatory mechanisms that can modulate the metabolic pathways of the fatty acids, leading to the production of specific lipid mediators. Moreover, also the relations, or the competition, between different metabolic ways might affect the productions of active metabolites, thus influencing the final effect.

In conclusion, this study sets out the steps to further investigate, in children as well in adults, the metabolism of dietary fatty acids, especially via CYP450, and the possible influence on hemodynamics and blood pressure control.

Supplemental Material Study 2

Supplemental Table S1: general characteristics and eicosanoid profile in obese children devided according to pubertal status.

	Post-Pubertal (n: 32)	**Pre-Pubertal (n: 38)**	
Variable	**Mediana (Range)**	**Mediana (Range)**	**p values**
BMI, Kg/m^2	29.85 (23.1;42.7)	28.21 (23.92;38.43)	0.23
z-score BMI	2.06 (1.3;2.8)	2.29 (1.64;3.29)	0.23
Waist, cm	97 (83;119)	91 (79;122)	0.26
z-score Waist	1.87 (0;2.4)	2.04 (1.41;3.5)	**0.01**
cIMT, mm	0.44 (0.3;0.6)	0.46 (0.33;0.56)	0.23
z-score cIMT	1.19 (-2;3.7)	2.15 (-2.22;4.95)	0.09
DC, 10^{-3}/KPa	39.65 (19.5;56.2)	42.26 (19.71;63.1)	0.7
z-score DC	-1.23 (-4.2;0.5)	-1.19 (-4.12;0.26)	0.9
CC, mm^2/KPa	1.14 (0.6;1.8)	1.16 (0.59;1.65)	0.9
FMD %	7.9 (1.9;16)	6.6 (-1.1;13.6)	0.8
RI, %	55 (20.3;76)	63.33 (37.33;83.33)	0.1
SI, m/s	6.14 (4.7;9.1)	6.14 (4.73;8.44)	0.19
O-SBP, mmHg	122 (107.7;152.3)	116 (102;163)	0.09
z-score O-SBP	0.95 (-0.1;3.4)	0.87 (-0.13;5.21)	0.81
O-DBP, mmHg	67.5 (52;83.7)	66.67 (56.67;88.33)	0.81
z-score O-DBP	0.34 (-1.5;1.8)	0.42 (-0.88;2.06)	0.81
24-h SBP, mmHg	117 (100;130)	115 (102;136)	0.81
z-score 24h-SBP	0.03 (-1.7;2.7)	0.69 (-0.79;2.77)	0.26
24h-DBP, mmHg	65 (58;78)	67 (55;79)	**0.05**
z-score 24h-DBP	-0.38 (-1.8;2.1)	0.07 (-2.02;2.32)	0.45
day-SBP, mmHg	120 (102;138)	118 (104;142)	0.33
z-score day-SBP	0.21 (-1.8;2.9)	0.58 (-1.07;2.93)	0.62
day-DBP, mmHg	68 (61;85)	70 (58;84)	0.14
z-score day-DBP	-0.69 (-1.9;2.2)	-0.33 (-2.07;1.98)	0.72
night-SBP, mmHg	107 (95;128)	108 (92;124)	0.45
z-score night-SBP	0.55 (-0.7;3.1)	1.11 (-0.7;2.63)	**0.04**
night-DBP, mmHg	58 (48;67)	59 (51;73)	0.79
z-score night-DBP	0.42 (-1.3;1.6)	0.57 (-0.88;2.56)	0.42

Supplemental Table S1 - continued

	Post-Pubertal (n: 32)	Pre-Pubertal (n: 38)	
Variable	**Mediana (Range)**	**Mediana (Range)**	**p values**
EpOMEs, ng/mL	9.83 (4;22.3)	11.45 (4.91;24.94)	0.62
DiHOMEs, ng/mL	6.01 (2.3;24.7)	7.84 (3.11;44.93)	0.63
EETs, ng/mL	7.61 (4.6;13.6)	7.69 (4.55;13.51)	0.8
DHETs, ng/mL	4.03 (2.3;6.5)	4.03 (2.18;6.84)	0.98
EEQs, ng/mL	0.34 (0.2;1.6)	0.36 (0.17;1.22)	0.8
DiHETEs, ng/mL	0.87 (0.5;3)	0.92 (0.52;3.55)	0.78
EDPs, ng/mL	3.12 (1.8;6.9)	2.9 (1.79;7.01)	0.82
DiHDPAs, ng/mL	0.98 (0.7;1.5)	0.82 (0.58;1.83)	**0.02**

BMI: body mass index; waist circ: waist circumference; cIMT: carotid intima-media thickness; DC: carotid distensibility; CC: carotid compliance; FMD: flow-mediated dilation; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure; DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid.

*Wilcoxon-Mann-Whitney U test.

Supplemental Table S2. Correlation between eicosanoids and z-score of blood pressure in the total population

Total Population								
	Z-Score SBP	**Z-Score DBP**	**Z-Score 24h-SBP**	**Z-Score 24h-DBP**	**Z-Score Day-SBP**	**Z-Score Day-DBP**	**Z-Score Night-SBP**	**Z-Score Night-DBP**
EpOMEs	-0.067	0.098	0.011	0.087	0.022	0.093	-0.044	0.038
DiHOMEs	0.032	**0.359^** 9.10-DiHOME **0.375^**	0.133	0.179	0.043	0.077	0.193	**0.309*** 9.10-DiHOME **0.312***
EETs	0.091	0.036	0.036	0.061	0.029	0.059	0.001	0.006
DHETs	0.152	0.077	0.056	0.036	0.090	0.081	-0.012	-0.020
EEQs	-0.115	-0.110	0.101	0.061	0.099	0.052	0.083	-0.019
DiHETEs	-0.065	-0.058	0.149	0.129	0.191	0.087	0.098	0.131
EDPs	-0.135	-0.114	-0.018	-0.040	-0.024	-0.031	-0.068	-0.067
DiHDPAs	-0.137	-0.091	-0.131	-0.065	-0.033	-0.021	-0.184 16.17-DiHDPA **-0.261***	-0.118
22-HDHA	-0.089	-0.092	-0.135	-0.183	-0.165	-0.163	-0.104	-0.236
20-HETE	0.199	**0.279***	0.003	0.059	-0.103	-0.51	0.132	0.153

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI.

DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Supplemental Table S3. Correlation between eicosanoids and vascular tests in the total population

Total Population							
	cIMT	**Z-Score cIMT**	**DC medio**	**Z-Score DC**	**RI**	**SI**	**FMD %**
EpOMEs	0.056	0.067	0.057	0.076	-0.023	0.065	-0.159
DiHOMEs	-0.089	-0.060	0.055	0.044	**0.402^** 9.10-DiHOME **0.382*** 12.13-DiHOME **0.392^**	0.231	-0.114
EETs	0.082	0.107	0.019	-0.015	-0.045	0.022	-0.175
DHETs	-0.063	-0.015	-0.081	-0.081	-0.087	0.049	-0.037
EEQs	-0.183	-0.196 14.15-EEQ **-0.241***	-0.014	0.035	-0.138	-0.104	-0.008
DiHETEs	0.000238	0.005	-0.028	-0.020	-0.042	-0.110	0.191
EDPs	-0.079	-0.086	0.080	0.088	-0.150	-0.152	-0.159
DiHDPAs	-0.152	-0.167	0.065	0.093	-0.075	0.039	-0.093 7.8-DiHDPA **0.250***
22-HDHA	-0.229	**-0.246***	-0.041	-0.036	-0.086	-0.132	-0.170
20-HETE	-0.009	0.046	-0.101	-0.125	0.123	0.142	-0.160

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI. DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; cIMT: carotid intima-media thickness; DC: carotid distensibility; FMD: flow-mediated dilation; RI: reflextion index; SI: stiffness index.

Supplemental Table S4. Correlation between fatty acids and z-Scores of blood pressure in hypertensive and normotensive population.

Total population								
	z-Score SBP	**z-Score DBP**	**z-Score 24-h SBP**	**z-Score 24-h DBP**	**z-Score Day-SBP**	**z-Score Day-DBP**	**z-Score Night-SBP**	**z-Score Night-DBP**
Omega-6 FA	0.14	0.21	0.05	0.03	-0.03	0.02	0.07	-0.08
LA	-0.03	0.18	0.22	0.20	0.19	0.13	0.21	0.16
AA	0.01	-0.10	-0.11	-0.16	-0.10	-0.07	-0.15	**-0.341^**
DGLA	0.17	0.18	0.09	0.13	0.07	0.06	0.07	0.14
Omega-3 FA	-0.08	-0.21	-0.16	-0.15	-0.06	-0.10	-0.06	-0.03
ALA	-0.04	0.12	0.04	0.06	0.04	0.08	0.05	0.03
EPA	-0.04	-0.16	0.01	-0.10	0.08	-0.09	0.05	0.04
DHA	-0.09	-0.21	-0.19	-0.17	-0.11	-0.13	-0.11	-0.04
Hypertensive								
	z-Score SBP	**z-Score DBP**	**z-Score 24-h SBP**	**z-Score 24-h DBP**	**z-Score Day-SBP**	**z-Score Day-DBP**	**z-Score Night-SBP**	**z-Score Night-DBP**
Omega-6 FA	0.346	0.323	-0.030	-0.220	-0.141	-0.249	0.113	-0.135
LA	0.042	0.247	-0.265	-0.195	-0.257	-0.263	-0.168	-0.220
AA	0.104	0.036	0.340	-0.214	0.263	-0.110	0.400	-0.253
DGLA	0.100	-0.110	0.125	-0.009	0.071	-0.156	0.102	0.185
Omega-3 FA	-0.174	-0.352	0.271	-0.119	0.267	-0.003	0.125	-0.125
ALA	-0.184	-0.187	-0.183	0.093	-0.146	0.077	-0.136	0.197
EPA	**-0.576***	**-0.648^**	0.036	-0.271	0.079	-0.188	0.002	-0.382
DHA	-0.158	-0.224	0.218	-0.073	0.238	0.013	0.035	-0.088
Normotensive								
	z-Score SBP	**z-Score DBP**	**z-Score 24-h SBP**	**z-Score 24-h DBP**	**z-Score Day-SBP**	**z-Score Day-DBP**	**z-Score Night-SBP**	**z-Score Night-DBP**
Omega-6 FA	0.112	0.220	0.202	0.168	0.081	0.174	0.146	0.042
LA	-0.139	0.148	0.232	**0.292***	0.166	0.247	0.249	0.251
AA	0.081	-0.091	0.009	-0.153	-0.025	-0.059	-0.154	**-0.333***
DGLA	0.204	0.151	0.015	0.073	-0.002	0.007	0.097	0.083
Omega-3 FA	-0.083	-0.097	-0.287	-0.160	-0.128	-0.115	-0.163	-0.029
ALA	-0.019	0.270	-0.008	-0.098	0.005	0.115	0.056	0.030
EPA	0.005	-0.051	-0.143	-0.130	-0.069	-0.114	-0.042	-0.027
DHA	-0.096	-0.120	**-0.315***	-0.188	-0.173	-0.161	-0.212	-0.035

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The underlined values represent the correlations that remain significant after adjustment for sex, age and BMI. AA: Arachidonic acid; ALA: alpha-Linolenic; DGLA: dihomo-gamma-linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LA: Linoleic acid; Omega-3 FA are calculated as the sum of ALA, EPA, Docosapentaenoic acid (DPA) and (DHA); Omega-6 FA are calculated as the sum of LA, GLA, DGLA, AA, Docosatetraenoic acid (DTA),Eicosadienoic acid and C22:5 ω6; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Supplemental Table S5. Correlations between z-score of blood pressure with vascular tests in the general population, in hypertensive and in normotensive subjects

	Z-score O-SBP	**Z-score O-DBP**	**Z-score 24h-SBP**	**Z-score 24h-DBP**	**Z-score Day-SBP**	**Z-score Day-DBP**	**Z-score Night-SBP**	**Z-score Night-DBP**
Total Population								
cIMT	0.13	-0.05	0.2	0.08	0.14	-0.05	0.24	0.17
z-Score cIMT	0.14	0.01	**0.264***	0.14	0.18	0.001	**0.289***	0.18
DC	**-0.549^**	**-0.258***	**-0.312***	-0.23	**-0.293***	-0.15	**-0.376^**	**-0.293***
z-Score DC	**-0.560^**	**-0.310***	**-0.334^**	**-0.252***	**-0.290***	-0.16	**-0.409^**	**-0.292***
FMD	-0.09	-0.06	-0.1	0.08	-0.05	0.11	-0.11	0.09
RI	-0.13	0.09	-0.01	0.03	-0.04	-0.04	-0.01	0.06
SI	-0.09	0.2	-0.18	0.14	-0.17	0.11	-0.23	0.06
Hypertensive								
cIMT	0.318	0.079	-0.055	-0.039	-0.95	-0.134	0.136	0.174
z-Score cIMT	0.171	-0.014	-0.132	-0.061	-0.161	-0.182	0.103	0.143
DC medio	**-0.745^**	0.086	-0.354	0.086	**-0.305***	0.042	-0.049	-0.022
z-Score DC	**-0.701^**	0.103	-0.371	0.046	-0.358	-0.007	-0.005	-0.005
FMD	-0.055	-0.108	0.2	0.437	**0.367^**	**0.578***	-0.258	0.247
RI	-0.046	-0.253	-0.358	-0.064	-0.345	-0.064	-0.33	-0.297
SI	-0.44	0.176	-0.46	0.42	-0.315	0.4	**-0.765^**	0.118
Normotensive								
cIMT	0.014	-0.187	0.163	0.133	0.112	-0.024	0.162	0.187
z-Score cIMT	0.02	-0.109	0.265	0.204	0.187	0.046	0.224	0.211
DC	**-0.494^**	-0.228	-0.252	-0.212	-0.239	-0.111	**-0.365***	-0.281
z-Score DC	**-0.500^**	**-0.300***	-0.265	-0.222	-0.213	-0.119	**-0.397^**	-0.268
FMD	-0.006	-0.034	-0.101	-0.007	-0.12	-0.041	0.002	0.055
RI	-0.259	0.078	-0.048	-0.025	-0.075	-0.083	-0.05	0.125
SI	-0.083	0.145	-0.249	0.029	-0.228	-0.009	-0.205	-0.005

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The underlined values represent the correlations that remain significant after adjustment for sex, age and BMI. cIMT: carotid intima-media thickness; DC: carotid distensibility; CC: carotid compliance; FMD: flow-mediated dilation; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Normotensive children:

Correlations of blood pressure with vascular tests

DC and the relative z-score were inversely correlated with office SBP and its z-score, with office DBP and its z-score, with 24-hours SBP, daytime SBP, nighttime SBP and its z-score and with nighttime DBP. RI was inversely correlated with office SBP and SI with 24-h SBP. IMT and its z-score directly correlated with nighttime SBP and nighttime DBP. (The correlations of blood pressure with vascular tests are detailed in Table 3. and correlations of z-score of blood pressure with vascular tests are detailed in Supplemental Table S2)

Correlations of erythrocyte membranes FA with blood pressure and vascular tests

In normotensive children AA showed an inverse correlation with BP, in particular nighttime BP, and an inverse correlation with RI. Also DHA was inversely correlated with RI. (Correlations of FA with BP values are detailed in Table 4 and those of the z-score of BP in Supplemental Table S3. Correlations of vascular tests are presented in Table 5)

Correlations of CYP450/sEH – derived eoicosanoids with blood pressure and vascular tests

In normotensive children 8,9-EEQs and 11,12-DiHETE showed several inverse correlations with DBP. EDPs inversely correlated with z-score of SBP, in particular office measurement. We found also a direct correlation of DiHOMEs with DBP. EDPs inversely correlated with the z-score of cIMT. RI was directly correlated with DiHOMEs and inversely with 5,6-EET and 5,6-DHET. (Correlations of eicosanoids with BP values are detailed in **Supplemental Table S6** and those of the z-score of BP in **Supplemental Table S7**, Correlations of vascular tests are presented in **Supplemental Table S8**)

Supplemental Table S6. Correlations between eicosanoids and blood pressure measurements in normotensive children

Normotensive								
	Office SBP	**Office DBP**	**24h-SBP**	**24h-DBP**	**Day-SBP**	**Day-DBP**	**Night-SBP**	**Night DBP**
EpOMEs	-0.031	0.187	0.107	0.184	0.112	0.235	-0.002	0.027
DiHOMEs	-0.080	**0.313*** 9.10-DiHOME **0.349***	0.072	0.197	-0.007	0.099	0.187	**0.357^** 9.10-DiHOME **0.355^** 12.13-DiHOME **0.309***
EETs	0.074	0.047	0.020	-0.028	-0.006	-0.023	-0.070	-0.188
DHETs	0.200 5,6-DHET **0.279***	0.135	0.019	0.045	0.054	0.065	-0.037	-0.005
EEQs	-0.057	-0.085 8.9-EEQ **-0.331***	-0.018	0.026	-0.020	0.027	-0.049	-0.197
DiHETEs	-0.040	-0.150 11.12-DiHETE **-0.293***	0.049	0.032	0.069	-0.044	-0.034	-0.016
EDPs	-0.136	-0.042	-0.032	-0.037	-0.015	-0.035	-0.195	-0.187
DiHDPAs	-0.002	0.008	-0.076	-0.052	-0.037	-0.058	-0.252	-0.117
22-HDHA	0.024	0.041	-0.031	-0.084	-0.072	-0.108	-0.027	-0.141
20-HETE	0.134	**0.285***	0.063	0.134	-0.050	0.001	0.157	0.258

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI. DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Supplemental Table S7. Correlation between eicosanoids and z-scores of blood pressure in normotensive children

Normotensive								
	Z-Score SBP	**Z-Score DBP**	**Z-Score 24h-SBP**	**Z-Score 24h-DBP**	**Z-Score Day-SBP**	**Z-Score Day-DBP**	**Z-Score Night-SBP**	**Z-Score Night-DBP**
EpOMEs	-0.037	0.180	0.055	0.178	0.110	0.229	-0.063	0.050
DiHOMEs	0.045	**0.405*** 9.10-DiHOME **0.423^**	0.141	0.193	0.024	0.086	0.250	**0.389^** 9.10-DiHOME **0.387^** 12.13-DiHOME **0.334^**
EETs	0.016	-0.037	-0.110	-0.058	-0.086	-0.041	-0.213	-0.174
DHETs	0.201	0.089	-0.017	0.046	0.015	0.068	-0.106	-0.059
EEQs	-0.091	-0.079	-0.055	0.013	-0.032	0.027	-0.098	-0.210
DiHETEs	-0.092 11.12-DiHETE **-0.298***	-0.119	-0.042	0.001	0.031	-0.053	-0.113	-0.053
EDPs	**-0.283*** 13.14-EDP **-0.316^** 19.20-EDP **-0.311***	-0.129	-0.220	-0.057	-0.130	-0.026	**-0.367*** 7.8-EDP **-0.367*** 10.11-EDP **-0.341*** 13.14-EDP **-0.361^** 16.17-EDP **-0.313*** 19.20-EDP **-0.409***	-0.159
DiHDPAs	-0.202	-0.119	**-0.289***	-0.063	-0.156	-0.032	**-0.379*** 7,8-DiHDPA **-0.381^** 10,11-DiHDPA **-0.378^** 16.17-DiHDPA **-0.401***	-0.160
22-HDHA	-0.002	-0.014	-0.098	-0.096	-0.136	-0.101	-0.046	-0.201
20-HETE	0.167	0.265	-0.008	0.113	-0.112	0.029	0.188	0.225

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI. DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; O-SBP/DBP: office systolic/diastolic blood pressure; 24h-SBP/DBP: 24-hours systolic/diastolic blood pressure; day-SBP/DBP: daytime systolic/diastolic blood pressure; night-SBP/DBP: nighttime systolic/diastolic blood pressure.

Supplemental Table S8. Correlations between eicosanoids and vascular tests in normotensive children

Normotensive							
	cIMT	**Z-Score cIMT**	**DC**	**Z-Score DC**	**RI**	**SI**	**FMD**
EpOMEs	0.056	0.058	0.041	0.071	-0.031	-0.048	-0.246
DiHOMEs	-0.194	-0.164	0.123	0.123	**0.352*** 9,10-DiHOME **0.306*** 12,13-DiHOME **0.363***	0.106	-0.247
EETs	0.125	0.089	-0.017	-0.115	-0.204	0.021	-0.234
DHETs	-0.031	-0.016	-0.100	-0.258	-0.275	0.008	-0.173
EEQs	-0.199	-0.247	-0.047	-0.020	-0.122	-0.075	-0.058
DiHETEs	0.001	-0.035	-0.082	-0.046	-0.164	-0.171	0.191
EDPs	-0.143	-0.213 16,17-EDP **-0.290*** 19,20-EDP **-0.305***	0.096	0.124	-0.182	-0.072	-0.096
DiHDPAs	-0.191	-0.262 10,11-DiHDPA **-0.290***	0.081	0.028	-0.195	0.034	-0.105
22-HDHA	-0.250	-0.268	-0.100	-0.117	-0.026	-0.058	-0.261
20-HETE	0.0001	0.030	-0.151	-0.168	0.079	0.228	-0.044

* : significance below 0.05; ^: significance below 0.01. In the table are reported the r_S values of the correlations. The correlations refer to the total amount of each ecosanoid acid. Singular isomers that are significant are reported below each correlation value. Underlined correlations are significant after adjustment for sex, age and BMI.

DHETE: dihydroxyeicosatrienoic acid; DiHDPA: dihydroxydocosapentaenoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHOME: dihydroxyoctadecenoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EET: epoxyeicosatrienoic acid; EpHOME: epoxyoctadecenoic acid; HDHA: hydroxydocosahexaenoic acid; cIMT: carotid intima-media thickness; DC: carotid distensibility; FMD: flow-mediated dilation; RI: reflextion index; SI: stiffness index.

Study 3: Blood pressure, fat distribution and vascular function in relation to polyunsaturated fatty acids in erythrocytes membranes, in children of primary schools: preliminary data

Introduction Study 3

A sedentary lifestyle and an unbalanced diet are the principal determinants of the onset of obesity in childhood as well as in adulthood. Longitudinal studies in children have documented that low levels of physical activity are unfavorably associated with the body composition[301–303].

Anyhow the suggested targets, i.e. 60 minutes per day of physical activity (http://www.who.int/dietphysicalactivity/factsheet_young_people/en/), are reached by a minority of children, with various percentage according to the different ages[304]. On the other side, a diet rich in fat, in particular saturated fatty acids, is associated with a higher cardiovascular risk[305]. Moreover, a reduction in polyunsaturated fatty acids (PUFA) and an increased omega-6 to omega-3 PUFA ratio are correlated with a further increase in cardiometabolic risk, due to a worsening in insulin-sensitivity and a poorer plasma lipid profile[306]. The prevalence of obesity varies in the different Countries and in different age groups, because also to the different definitions and cut-off point available. In Italy it has been estimated that 12% children aged 8-9 year are overweight and 22.2% obese, whereas the prevalence of severe obesity, based on WHO definition, is 4.5%[11]. A cross-sectional survey of childhood obesity was performed on 3923 children aged 6-11 years from 19 Italian schools. About 27% of boys and 25% of girls were overweight and the prevalence of obesity was almost the same (21%) in boy and in girls. The prevalence of hypertension was 9.9% in boys and 13.9% in girls[15].

In this study we aimed to investigate the prevalence of body weight excess and elevated blood pressure in a sample of children of the 3rd and 4th classes of primary schools in Verona. We further investigated the influence of physical activity and dietary habits, in particular the intake of fatty acids, on weight excess, blood pressure and some indexes of vascular function.

Material and methods Study 3

Children were recruited from the 3rd and 4th classes of three primary schools of Verona South district. Inclusion criteria were the followings: children of the abovementioned classes who accepted to participate in the study and whose parents gave a written informed consent. Exclusion criteria were either the lack of written informed consent signed by the parents or refusal to participate by the child.

Study design

The study was conducted according to a cross-sectional observational design. The study was approved by the Ethical Committee of Verona (CESC n. 375).

Assessment

Children were evaluated in the morning from 8.00 a.m to 1 p.m. Fasting was not required. Two questionnaire were collected: a validated food frequency questionnaire (FFQ), integrated with specific questions about PUFA

intake[307,308] and a validated physical activity questionnaire (PAQ), integrated with semiquantitative data to calculate the weekly metabolic equivalent (METs) as presented in the international PAQ (iPAQ) [309–311].
The questionnaires were previously given and explained to the children and they were requested to fill it in with the aid of parents. All the questionnaires were then accurately checked by a dedicated dietician together with the child.
Then, the participants underwent a physical examination with the collection of anthropometric measurements. Body weight, height, and waist and hip circumferences were measured with the patient wearing light clothes. Body weight was measured by a calibrated balance and height by a calibrated stadiometer. Body mass index (BMI) was calculated as weight in Kg divided by the square of height in m; overweight or obesity were defined for BMI≥90^{th} and 95^{th} percentile for sex and age, respectively[255]. WHO reference for BMI was used for categorizing children into the overweight and obese groups[2]. Waist/hip ratio was calculated as waist circumference in cm divided by hip circumference in cm and waist/height ratio (WHtR) was calculated as waist circumference in cm divided by height in cm. Waist circumference was transformed in z-score and percentile according to normative values[260]. Through a bioelectrical impedance analysis (Tanita MC 780 MA) was obtained an estimation of body composition and in particular an estimation of fat (%) and fat mass (FM, Kg), fat-free mass (FFM, Kg), total body water (TBW, Kg) and the basal metabolic rate (BMR, KJ and Kcal). During the visit blood pressure was measured by a semiautomatic oscillometric device with a specific validation for children (Omron 705 IT), [312], at least 3 times, 3 minutes apart with the patient lying supine for at least 10 minutes before the first measurement in a room with controlled temperature (22-24°C). The mean of the 3 BP measurements were transformed in z-score and percentile, according to normative values and current guidelines [258,313]. The 95^{th} of office BP measurements was used as cut-off for hypertension, according to current European guidelines[26].

Vascular tests
Some indexes of systemic vascular stiffness, i.e. *Stiffness Index* (SI) and *Reflexion Index* (RI), were estimated by Digital Volume Pulse (DVP) method and were obtained with the digital photoplethysmography PulceTrace PT 1000 (MicroMEdical Ltd, Gillingham, Kent, UK) as previously described[292].
Measures of the central arterial *pressure waveform* and *pulse wave velocity* as well as an assessment of arterial stiffness via waveform analysis (e.g., augmentation index, augmented pressure) were provided by SphygmoCor XCEL.
To conduct a carotid-femoral PWV measurement, a cuff was placed around the femoral artery of the patient to capture the femoral waveform, and a tonometer was used to capture the carotid waveform. The distance between the carotid and femoral arteries was measured, and the velocity automatically determined by dividing the distance by the pulse transit time. The relative z-score and percentile were calculated according to reference values[314].

Laboratory measurements
At 12 a.m., after at least 4 hours of fast, a few blood drops, in willing children, were collected by a fingerprick for plasma cholesterol, triglycerides and glucose

measurement, using an automatic point-of-care testing instruments (HPS MultiCare-in, Italy), as previously described [315].

For *erythrocyte membrane fatty acid analysis*, a single drop of scavenged whole blood was collected directly to a filter paper (Ahlstrom 226, PerkinElmer, Greenville, SC) that was pretreated with an antioxidant cocktail (Oxystop, OmegaQuant Analytics, LLC, Sioux Falls, SD) to protect unsaturated FAs from oxidation. After collection, cards were stored in a re-sealable plastic bag delivered immediately to Omegametrix GmbH (Martinsried, Germany) for analysis by capillary gas chromatography as described previously [316,317]. Fatty acid levels are expressed both as a weight percent of total blood fatty acids (composition). The stability of FAs collected and stored in this manner has been previously evaluated and no sample degradation was detected [318].

Estimation of Δ^9 , Δ^6 and Δ^5 desaturase activity

Δ^9 , Δ^6 and Δ^5 desaturase are enzymes responsible for the endogenous formation of monounsaturated and polyunsaturated FA and their activity has been associated with insulin-glucose homeostasis and with central obesity. We estimated the desaturase activity as the ratio of product to precursor of individual red blood cell membrane FA as follows: Δ^9-desaturase (SCD) = C16:1n-7/C16:0 and C18:n-9/C18:0 (they will be referred to as SCD-16 and SCD-18, respectively); Δ^6-desaturase (D6D) = C18:3n-6/C18:2n-6 and Δ^5-desaturase (D5D) = C20:4n-6/C20:3n-6.[265,278]

Follow-up of children with elevated BP

The children who had elevated BP at the first evaluation were requested to undergo a medical examination that included a new height measurement, as above described, and BP measurement by the same semiautomatic oscillometric device (Omron 705 IT) as in the first visit. Blood pressure was measured in both arms, then in the arm with higher BP levels, BP measurements were repeated 5 times, 3 minutes apart, with the children in the sitting position, in a quiet and tempered room, after at least 10 minutes resting. The mean of the last two measurements were then calculated and transformed in z-score and percentile according to current guidelines[313]. Than a BP measurement by a manual anaeroid device was obtained to confirm the oscillometric measurements. Children were advised to avoid strenuous physical activity before the medical examination.

STATISTICS

Data are presented as the mean ± standard deviation unless otherwise stated. The statistical analysis was performed using the software Statistical Package for Social Sciences software (SPSS / PC for Windows version 21.0). Bivariate parametric correlations were estimated by Pearson coefficient (r).

Differences in the measured parameters between groups were analyzed by parametric (T-student test). A two-tailed test with a $p<0.05$ was considered statistically significant.

Preliminary results Study 3

We enrolled 309 children, aged 8.64 ± 0.7 year, from March 2016 to May 2017 in 3 primary schools of Verona. Hundred fifty-five were female (50%). General characteristics of the children are detailed in **Table 1**.

All children underwent anthropometric measurements; vascular tests were performed in 303 children; 244 children accepted to undergo blood drop collection and FA analysis, as well glucose measurements, were obtained in 244 children, whereas cholesterol was measured in 183 children and triglycerides in 202 subjects. Results are missing in children who did not give the consent for blood drop or for technical reason (not enough blood to perform all the measurements).

No significant differences of anthropometric parameters were found between males and females, except a higher body content of fat in girls (fat: 19 ± 8.3% *vs* 22.3 ± 9.5%, $p < 0.01$ and fat mass: 7.2 ± 5.2 Kg *vs* 8.5 ± 5.7 Kg, $p < 0.05$, respectively). On the basis of the percentile of BMI, 19% (n: 60) of children were overweight and 13% (n: 42) were obese, without significant differences between boys and girls, even if the first showed a slightly higher prevalence of overweight and obesity in comparison to girls (overweight 20% vs 17% and obese 14% vs 12%, $p > 0.05$, respectively) (**Figure 1**).

Waist/height ratio, an index of central distribution of fat, was below 0.5 in 93% of normal-weight children, 48% in overweight children and 12% in obese children (**Figure 2**).

When considering the subgroup of children with weight excess (obese + overweight) in comparison to the normal weight children, they showed higher SBP (including the z-score) and a higher percentile of PWV, which was anyhow within the normal range. The weight excess group had lower levels of total erythrocyte membrane omega-3 PUFA and of Omega-3 Index. The others FA did not differ between the groups. Omega-3 Index was lower than the suggested cut-off point of 8% in 99.4% of the whole population and was lower than 4% in 52.96%. In normal weight children the prevalence of Omega-3 Index below 4% was 47.3%, it increased to 55.3% in overweight and to 64% in obese subjects (**Figure 3**). On the basis of the percentile of basal BP measurements in the whole population, 22% (n: 65) children resulted to have BP values in the range of hypertension (>95th percentile) and 17% (n: 54) in the range of normal-high BP (>90th percentile but <95th percentile) and the prevalence of high blood pressure tended to be higher in obese children: 31% (n: 13/42) of obese children had BP in the range of hypertension and 12% (n: 5) in the range of normal-high BP, without statistically significant differences with the whole population.

To date 25 willing children, who had BP in the range of hypertension at the basal visit, underwent the follow-up visit. At the re-evaluation of the children, after repeated BP measurements in standard conditions, only one child had BP levels in the range of hypertension and 2 children presented normal-high BP.

In the whole population, we found that SBP, DBP and central-DP were directly correlated with BMI, waist circumference, hip circumference and waist/height ratio, but the latter showed a weaker correlation with BP in comparison to the other anthropometric measurements. Even FM directly correlated with SBP, DBP and central-DP, whereas FFM was directly correlated with only SBP. When considering obese children, most correlations found in the whole population were significant and showed a higher strength. In particular, in obese subjects the direct correlation of waist/height ratio with BP was almost as strong as those of other anthropometric features and central-DP showed significant direct correlations with only waist circumference and waist/height ratio (The correlations of anthropometric features with BP and vascular tests are detailed in **Table 2** for the whole population and in **Table 3** for obese children, and showed in **Figure 4**).

PWV showed direct correlations with SBP and DBP in the whole population, whereas in obese children it was more strongly correlated with DBP and central DP.

In the *whole population* we found that Omega-3 PUFA and DHA showed a weak but significant inverse correlation with BMI ($r= -0.134$, $p<0.05$ and $r=-0.143$, $p<0.05$, respectively), waist/height ratio ($r=-0.153$, $p<0.05$) and the relative z-scores, and also Omega-3 Index was inversely correlated to waist/height ratio ($r=-0.134$, $p<0.05$), whereas GLA, an omega-6 FA, was directly correlated to z-score of BMI ($r=0.149$, $p<0.05$) and to waist/height ratio ($r= 0.160$, $p<0.05$). SFA and PA showed weak inverse correlation with BP (SFA - DBP: $r= -0.130$, $p<0.05$; PA - central-DP: $r= -0.126$, $p<0.05$) and with SI ($r= -0.153$, $p<0.05$ and $r= -0.150$, $p<0.05$, respectively). Omega-3 Index were also inversely correlated to plasma glucose and triglycerides ($r= -0.169$, $p<0.01$ and $r= -0.142$, $p<0.05$, respectively), as well as DHA. Total omega-6 PUFA were inversely correlated to triglycerides ($r= -0.215$, $p<0.01$) and also LA were inversely correlated, whereas omega-6 PUFA showed a direct correlation with total cholesterol ($r= 0.201$, $p<0.01$). On the other hand, GLA showed a direct correlation with total cholesterol and glucose ($r= 0.2169$, $p<0.01$ and $r= 0.158$, $p<0.05$, respectively).

The estimated activity of D5D showed an inverse correlation with waist circumference ($r= -0.128$, $p<0.05$), whereas D6D showed some weak correlations with waist/height ratio ($r=0.165$, $p<0.05$), plasma cholesterol and glucose ($r= 0.176$, $p<0.05$ and $r=0.172$, $p<0.01$, respectively) (**Figure 6**).

In *obese children* we found that omega-3 PUFA were inversely correlated with z-score of BMI ($r= 0.350$, $p<0.05$), whereas GLA and DGLA were directly correlated with some anthropometric measures (GLA-z-score BMI: $r=0.350$, $p<0.05$; DGLA-waist: $r= 0.402$, $p<0.05$; DGLA-waist/height: $r= 0.347$, $p>0.05$). In obese children total saturated FA (SFA) and palmitic acid (PA) were directly correlated with central-DP ($r= 0.402$, $p<0.05$ and $r= 0.357$, $p<0.05$, respectively); LA was directly correlated with SBP ($r= 0.342$, $p<0.05$). Omega-6 PUFA showed an inverse correlation with triglycerides ($r= -0.610$, $p<0.019$) and in particular LA was inversely correlated to triglycerides ($r= -0.389$, $p< 0.05$) and to plasma glucose ($r=-0.370$, $p<0.05$). The estimated activity of D5D showed inverse

correlations with waist circumference (r= -0.342, p<0.05) and with PWV (r= -0.358, p<0.01) (Figure 6).

Table 1. General characteristics of the children

Variable	Male (n: 154)	Female (n: 155)	p-value*	Obese-Overweight (n:102)	Normal-weight (n:204)	p-value*
BMI (Kg/m^2)	18,1±3,2	18,3±3,7	0,662	21,8±3,1	16,3±1,6	**0,0001**
Percentile BMI	63,8±30,1	61,8±32	0,575	93,7±4	46,4±26,2	**0,0001**
Waist(cm)	63,5±9	62,4±10,4	0,339	71,7±8,3	58,1±6,5	**0,0001**
Percentile Waist	56,8±31,2	55,6±31	0,740	85,1±15,8	40,5±25,4	**0,0001**
Waist/Height ratio	0,5±0,1	0,5±0,1	0,845	0,5±0,1	0,4±0,1	**0,0001**
Percentile W/H ratio	46,7±32	43,7±32	0,417	75,3±21,7	29,4±23,9	**0,0001**
Waist/Hip ratio	0,9±0,1	0,8±0,1	0,309	0,9±0,1	0,8±0,1	**0,002**
FAT	19±8,3	22,3±9,5	**0,002**	28,8±7,7	16,2±6,2	**0,0001**
FAT_MASS (kg)	7,2±5,2	8,5±5,7	**0,049**	12,9±5,9	5,1±2,5	**0,0001**
FFM (kg)	27±4,9	26,3±4,2	0,169	29,7±4,1	25±3,9	**0,0001**
TBW (kg)	19,9±3,2	19,2±3,1	0,084	21,7±3	18,4±2,6	**0,0001**
SBP (mmHg)	110,6±9,6	110,5±10,5	0,950	113±8,6	109,2±10,5	**0,001**
Percentile -SBP	75,9±20,7	76,1±20,5	0,936	81±17,8	73,4±21,4	**0,002**
DBP (mmHg)	66,4±7,6	67,1±8,1	0,428	67,5±7,7	66,3±7,9	0,199
Percentile -DBP	70,1±19,8	72±19,7	0,386	71,9±19,2	70,6±20,1	0,572
Central-DP (mmHg)	70,2±11,7	71,1±9,3	0,476	71,5±8,8	70,3±11,4	0,333
RI (%)	77,7±11	73,5±13,3	**0,003**	71,6±12,9	77,9±11,5	**0,0001**
SI (m/s)	6,2±1,3	6,6±1,9	0,073	6,3±1,3	6,5±1,8	0,407
AIx (%)	6,9±13,6	9,5±12,9	0,097	7,9±13,1	8,4±13,4	0,787
PWV (m/s)	4,7±1,5	4,6±0,9	0,778	4,8±1	4,6±1,4	0,213
Glucose (mg/dl)	92,2±8,9	85,7±10,4	**0,0001**	89,3±9,4	87,8±10,5	0,270
Cholesterol (mg/dl)	241,5±36,2	220,8±38,2	**0,0001**	228,15±40,4	231,9±37,7	0,534
Triglyceride (mg/dl)	176,8±83,5	164,8±62,8	0,247	192,5±81,7	159,2±65,8	**0,002**
PA, %	23,1±1,3	23±1,4	0,618	23,1±1,3	23±1,4	0,498
ALA, %	0,2±0,1	0,1±0,1	0,345	0,1±0,1	0,2±0,1	0,133
EPA, %	0,3±0,2	0,3±0,2	0,341	0,3±0,2	0,3±0,2	0,225
DHA, %	2,8±0,7	2,9±0,7	0,584	2,7±0,7	2,9±0,8	**0,013**
LA, %	19,9±2,3	19,8±2,8	0,924	19,7±2,2	19,9±2,8	0,523
GLA, %	0,2±0,1	0,2±0,1	0,375	0,2±0,1	0,2±0,1	**0,014**
DGLA, %	2±0,4	2±0,9	0,654	2±0,4	2±0,8	0,471
AA, %	12,3±1,9	12,1±2	0,334	12,4±1,9	12,1±1,9	0,362
Omega-3 Index, %	4±0,8	4±0,9	0,772	3,8±0,8	4,1±0,9	**0,015**
Ω-3 PUFA, %	4,3±1,2	4,2±1	0,476	4,1±0,9	4,3±1,2	**0,038**
Ω-6 PUFA, %	36,7±4,4	36,5±4,8	0,672	37±2,7	36,3±5,4	0,263
Ω-9 FA, %	19,3±2,6	19,9±3,1	0,132	19,8±2	19,5±3,3	0,329
***Trans* FA, %**	0,7±0,3	1±3,8	0,349	0,6±0,3	0,9±3,4	0,420
Saturated FA, %	24,9±2,8	24,8±2,8	0,782	25,1±1,5	24,7±3,3	0,303
Total PUFA, %	60,3±6,2	60,5±7,1	0,806	60,9±2,4	60,2±8,2	0,396
D5D	6.5±1.8	6.5±1.7	0.989	6.2±1.8	6.6±1.7	0.125
D6D	0.010±0.007	0.009±0.004	0.272	0.011±0.007	0.009±0.004	**0.007**

BMI: body mass index; FAT: FFM: free fat mass; TBW: total body water ; SBP: systolic blood pressure; DBP: diastolic blood pressure; Waist: waist circumference; RI: reflexion index; SI: stiffness index; Aix: augmentation index; PWV: pulse wave velocity; PA: palmitic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; GLA: gamma-linolenic acid; DGLA: dihomo-gamma-linolenic acid; AA: arachidonic acid; D5D: delta-5 desaturase; D6D: delta-6 desatutrase.

* Indipendent sample T-test

Figure 1. Prevalence of overweight and obesity in the whole population and in males and females

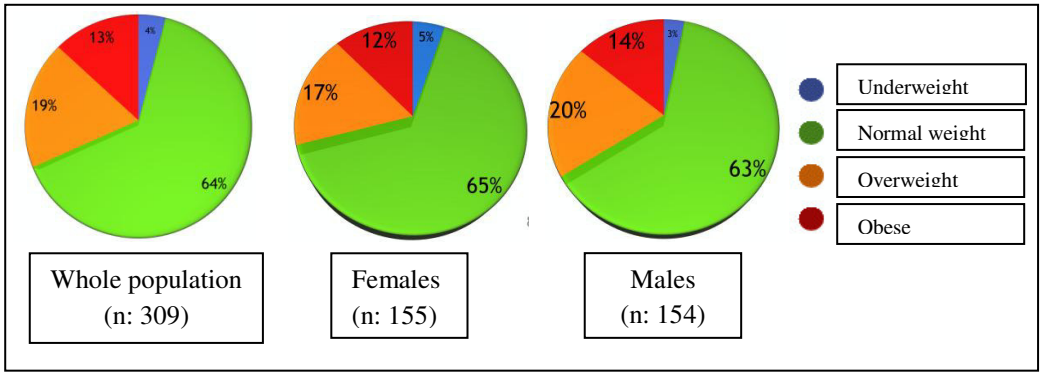


Figure 2. Distribution of waist/height ratio according to weight

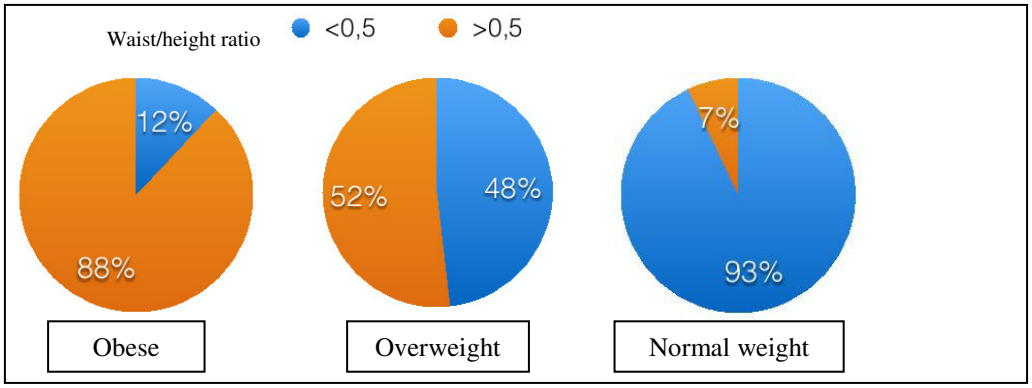


Figure 3. Omega-3 Index in the whole population and in the subgroups, divided according to gender and weight

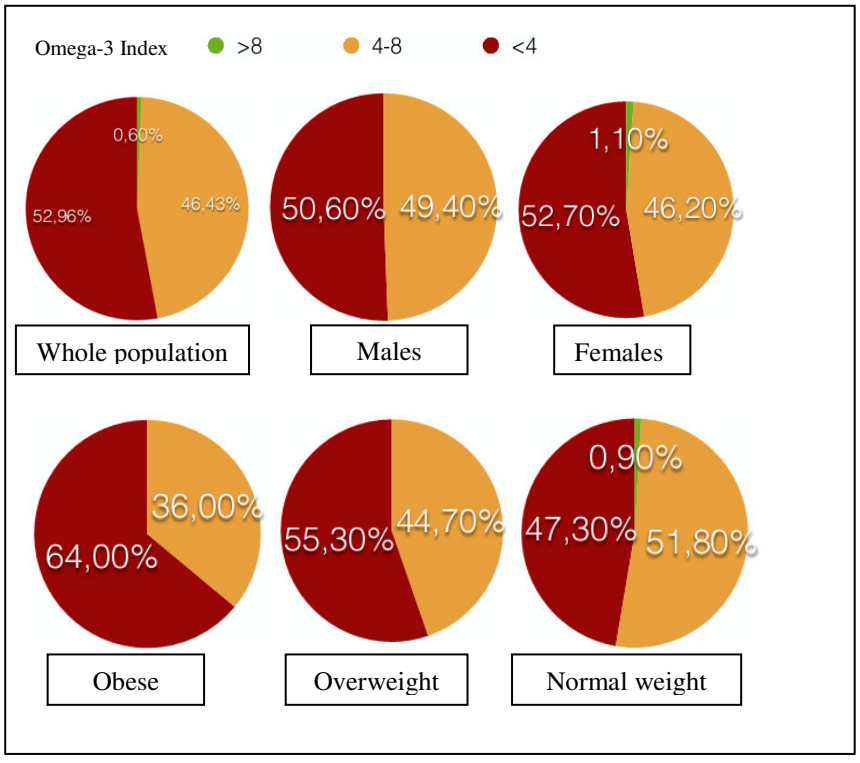


Table 2. Correlations of anthropometric parameters with BP and vascular tests in the whole population

	SBP	z-score SBP	DBP	z-score DBP	Central-DP	SI	PWV	z-score PWV
BMI	**0,291^**	**0,236^**	**0,158^**	0,089	**0,138***	-0,041	0,008	-0.024
z-score BMI	**0,257^**	**0,214^**	**0,133***	0,081	**0,126***	0,021	-0,030	-0.050
Waist	**0,285^**	**0,186^**	**0,157^**	0,070	**0,141***	-0,004	0,069	0.038
z-score Waist	**0,261^**	**0,176^**	**0,161^**	0,103	**0,140***	0,070	0,034	0.015
Waist/Height	**0,122***	0,092	0,099	0,070	0,065	0,010	-0,005	-0.003
z-score Waist/Height	**0,140***	**0,128***	**0,131***	**0,129***	0,076	0,028	-0,02	0.023
Hip	**0,335^**	**0,212^**	**0,155^**	**0,033**	**0,134***	0,018	0,061	0.012
Waist/Hip	0,017	-0,08	0,023	0,008	0,030	0,003	0,019	0.048
FM	**0,327^**	**0,237^**	**0,204^**	0,111	**0,191^**	-0,062	0,050	0.008
FFM	**0,368^**	**0,191^**	0,79	-0,084	0,110	0,003	0,101	0.032

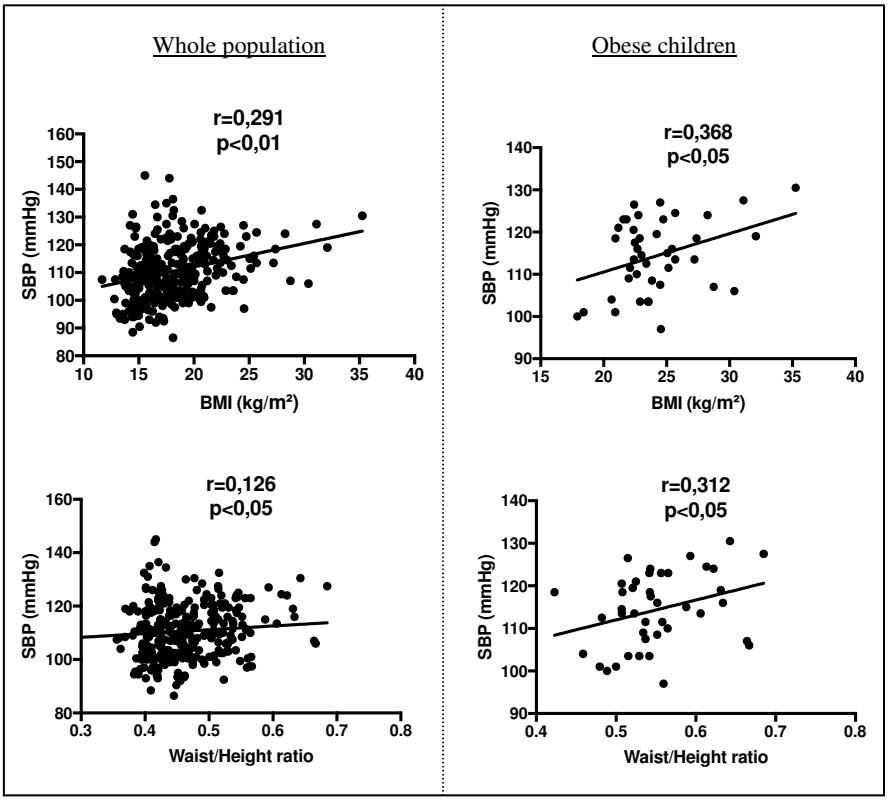
In the table are presented the r values of the correlations. ^: $p<0.01$; *: $p<0.05$ SBP: systolic blood pressure; DBP: diastolic blood pressure; Central DP: central diastolic pressure; BMI: body mass index; FM: fat mass; FFM: fat free mass; hip: hip circumference; waist: waist circumference; PWV: pulse wave velocity.

Table 3. *Correlations of anthropometric parameters with BP and vascular tests in obese children*

	SBP	z-score SBP	DBP	z-score DBP	Central-DP	SI	PWV	z-score PWV
BMI	**0,368***	**0,321***	**0,363***	**0,313***	0,275	-0,228	-0,021	-0.158
z-score BMI	0,067	0,087	0,043	0,055	0,051	-0,171	0,036	-0.055
Waist	**0,351***	**0,291**	**0,380***	**0,326***	**0,356***	-0,189	0,286	0.160
z-score Waist	**0,358***	**0,314***	**0,392***	**0,364***	**0,364***	-0,086	0,314	0.226
Waist/Height	**0,312***	**0,322***	**0,383***	**0,378***	**0,360***	-0,218	0,224	0.155
z-score Waist/Height	0,289	0,304	**0,360***	**0,364***	**0,374***	-0,181	0,283	0.222
Hip	**0,326***	**0,242**	0,253	0,172	0,178	-0,106	0,061	-0.102
Waist/Hip	0,127	0,140	0,243	0,263	0,296	-0,156	**0,331***	**0.352***
FM	**0,363***	0,292	**0,352***	0,270	0,220	-0,255	-0,108	-0.270
FFM	0,195	0,050	0,109	0,025	0,147	0,047	**0,327***	0.202

In the table are presented the r values of the correlations. ^: p<0.01; *: p<0.05 SBP: systolic blood pressure; DBP: diastolic blood pressure; Central DP: central diastolic pressure; BMI: body mass index; FM: fat mass; FFM: fat free mass; hip: hip circumference; waist: waist circumference; PWV: pulse wave velocity.

Figure 4. Correlations of anthropometric parameters and blood pressure in the whole population and in obese subjects



BMI: body mass index; SBP: systolic blood pressure.

Figure 5. Correlations of omega-3 and omega-6 PUFA in the whole population and in obese children.

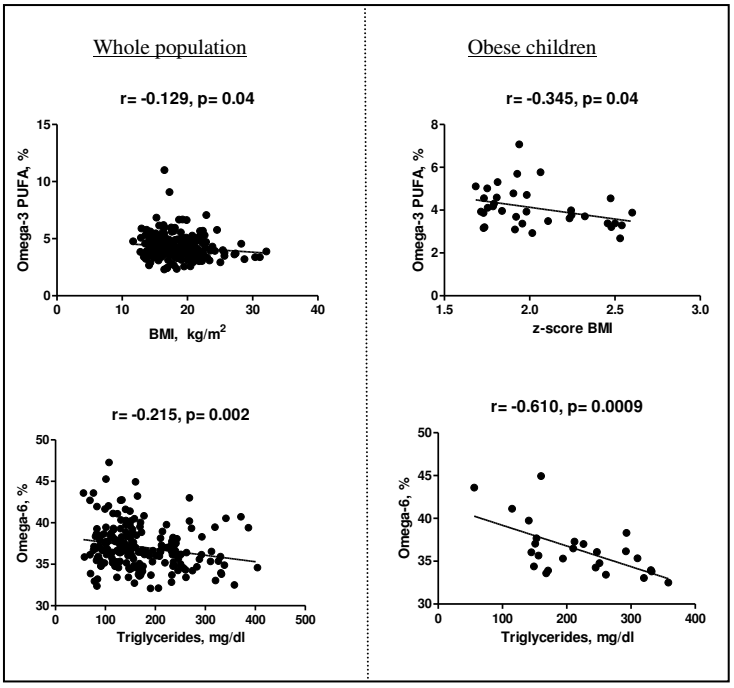
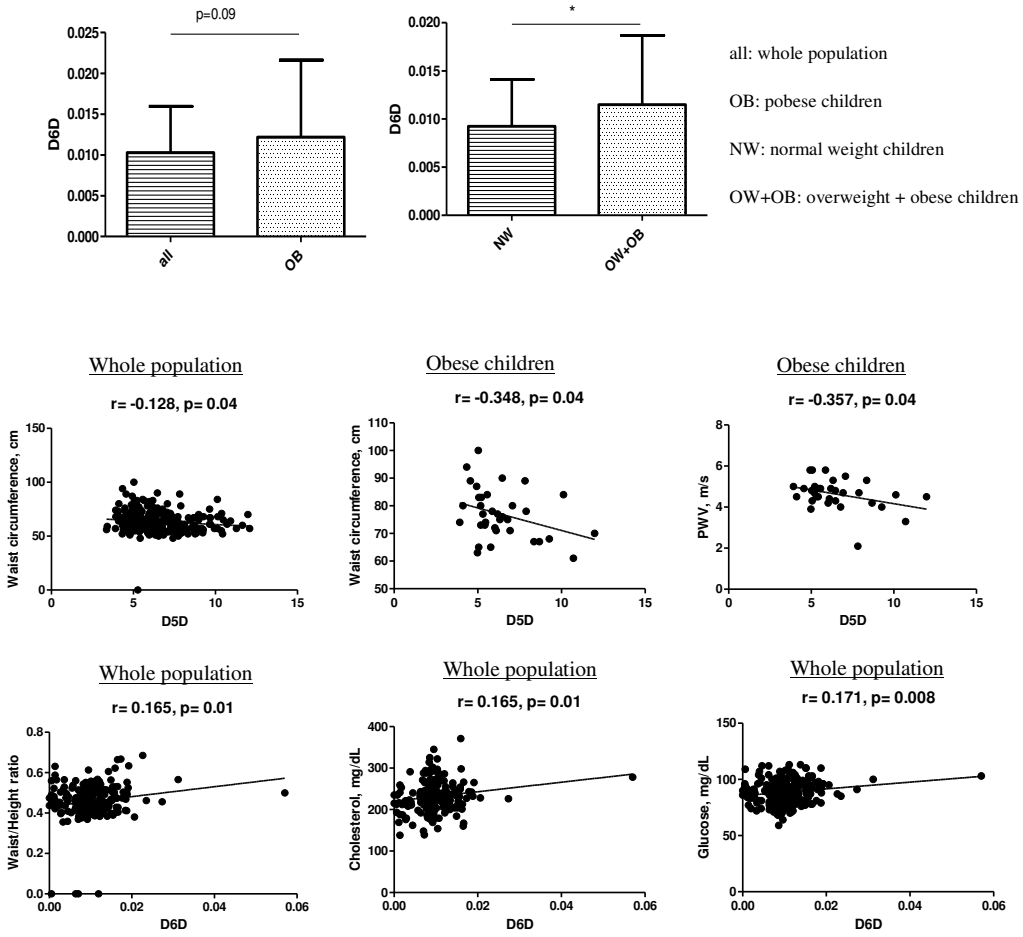


Figure 6. Comparison of Delta-5 and Delta-6 desaturase activity in different weight classes and their correlation in the whole population and in obese children



D5D: Delta-5 desaturase activity; D6D: Delta-6 desaturase activity; PWV: pulse wave velocity

Discussion Study 3

Even if the sample size is limited, our data confirm recent published prevalence of overweight and obesity in a similar aged population by the national survey "Okkio alla salute" [319]. Furthermore, our data indicate that the increase in BMI is accompanied by an increase in central distribution of the fat, which is associated with a higher cardiovascular risk. Children with weight excess showed also higher BP levels, in particular SBP, and a higher percentile of PWV, although in the normal range [314], but significantly elevated in comparison to normal-weight children. These data indicate an impact of weight excess on BP control and on arterial stiffness that are detectable also in the early childhood. When considering the different indexes of weight excess and fat distribution, stronger correlations of the markers of central obesity with BP and arterial stiffness were detectable in overweight and obese children rather than in normal weighted children. Moreover, also the bioelectrical impedance analysis confirms the results of the other anthropomentric measurements, indeed both fat mass and fat-free mass were correlated with BP in all children, whereas in obese children only fat mass was directly associated with BP.

In the analysis of erythrocyte membrane fatty acids, which are an index of mid-term dietary intake of fatty acids [320], we found a low level (nearly 4%) of omega-3 PUFA in the whole population and only 0.6% of children had an Omega-3 Index over 8%, which is considered to be associated with a CV protection [183]. Moreover, omega-3 PUFA were inversely associated with BMI and high levels of omega-3 PUFA were found only in normal weight children. These data indicate a low intake of fish by the diet, in particular in overweight and even more in obese children. Beyond the putative protective CV effect of omega-3 PUFA, these results may be a marker of an unbalanced diet, in particular a low intake of fish is generally assumed to be associated with lower vegetable and fruit intake and higher intake of meat, depicting unhealthier dietary habits, that are, together with physical activity, the main determinant of weight excess [239,240].

In our sample omega-6 PUFA, and LA in particular, showed an inverse correlation with triglycerides in the whole population, even stronger in obese subjects. This result can support the hypothesis of a protective role of omega-6 PUFA on cardiometabolic risk factors, according to the findings of our first study (Bonafini et al., unpublished data, see above) and other previous observation [189,190]. Anyhow in the sample analyzed in the present work, the other cardiovascular parameters were not associated with omega-6 PUFA neither in the whole population nor in the obese group.

Interestingly, within the omega-6 family, GLA seems to have an opposite association with the analyzed variables in comparison to the total amount of omega-6 PUFA, as we already found in the first presented study, and it was significantly higher in children with weight excess. Little is known about the action of GLA on metabolic profile, especially in children [276], and further investigation are needed in order to understand whether it might exert a role on metabolic homeostasis rather than being a marker of a poorer metabolic profile.

Indeed, GLA is the product of D6D starting from LA, and a higher activity of this enzyme has been linked to insulin sensitivity[279] and to a unfavourable metabolic status[265,277]. In our population we found that the level of estimated D6D activity were higher in overweight and obese children as compared to normal-weight subjects, and it was correlated with indexes of central obesity. Our data confirm the current knowledge about D6D, as well as the results of our first study (see above).

The enlargement of the sample size and the in-depth analysis of the food frequency questionnaires will give data also about different dietary patterns and their relationship with erythrocyte membrane fatty acids that will help to better understand the association of macronutrients with body composition and cardiovascular risk factors.

In this study we found a high prevalence of elevated blood pressure in children at the first visit but the re-evaluation of the children with high BP, in standard condition, demonstrated a low prevalence of "real hypertension" in children. In fact, BP levels have been probably influenced by the contingent circumstances rather than by a real hypertensive status and thus we confirm the suggestions of current guidelines to accurately evaluate BP measurements in children and the need of repeated measurements before making a diagnosis of hypertension, or even of white-coat hypertension [26]. Indeed, our data contradict other recent surveys, in larger population with comparable percentage of obese subjects, which showed a prevalence of hypertension in the range of 8%.

Anyhow, it is worthy of mention the finding of a positive association of weight excess with BP levels and arterial stiffness, which underlines the possible harmful effect of body weight and fat distribution on cardiovascular system, at least in a subclinical manner, in otherwise healthy children.

Our study has limitations: the cross- sectional design of the study do not allow to conclude for a causative link of the associated variables. Anyhow, some meaningful associations were found, partially confirming our previous findings, thus suggesting a possible role of PUFA regarding the metabolic status of the children. It is worth underlining that the analysis of FA pattern on a blood drop is not completely comparable with the analysis obtained from RBC membranes extracted from a blood sample. Indeed, the results derived from blood drops reflect not only the FA in RBC membranes but also the plasma composition, and is therefore more influenced by the fasting status. Caution is therefore needed in the interpretation and comparison of the results of study 2 and 3, which were anyway obtained by analysis using the actual gold standard method, namely capillary gas chromatography.

Moreover, as above mentioned, the number of children included in the BP follow-up was low, so the results cannot be interpreted as the prevalence of hypertension among school-aged children. However, they underline the importance of a correct BP measurements, in standard conditions and with repeated measurements, in order to prevent an overestimation of the real prevalence of hypertension.

In conclusion, our data confirm a high prevalence of obesity/overweight in young children (8-10 years) attending the primary school in Verona South district, but, at a more careful evaluation denies a high prevalence of hypertension. Still, the correlations between anthropometric indexes, especially central distribution of fat with BP and arterial elasticity, call for an action to modify unhealthy behavior already in children to prevent a possible progression over time of target organ damages.

The low intake of omega-3 PUFA seems to be one corrigible factor, but the lack of significant correlation with body fat, BP, elasticity and the very low correlation with triglycerides scales down their role as one of the major determinant of these risk factors. Also the putative role of omega-6 PUFA, which we previously found as inversely associated with several components of metabolic syndrome in a sample of obese children, is not completely confirmed in this collection, where only an inverse association with triglycerides were detectable. The specific role of GLA, which showed, at variance with other omega-6 but similarly to our previous study, a direct association with a worst metabolic profile, as well as the clinical effects of D5D and D6D activity, warrant further evaluations.

Final remarks

Taken together our data indicate that weight excess, whose prevalence is elevated even in young children, is associated with a worst metabolic profile, vascular function and BP control even if in the range of normality, as compared to normal weight children. The putative beneficial effect of omega-3 PUFA appears rather weak, even if both the investigated samples presented low levels of omega-3 PUFA, which might have blurred their biological effects. Then, the clinically relevant actions of their metabolites via CYP450/sEH on BP and vascular structure were detectable only in the subgroup of hypertensive obese children. Further investigations are needed to assess the stimuli and the conditions that can modulate these metabolic pathways, thus clarifying whether various lipid mediators can be involved in different clinical settings.

Our results suggest a protective effect of omega-6 PUFA, and in particular of arachidonic acid, on metabolic profile and BP control, but do not support the hypothesis that CYPP450 metabolites mediate these effects. The role of linoleic acid and its CYP450/sEH-derived metabolites, which were associated to BP, remains unclear, also because of the scanty evidences in humans and especially in children. Interestingly, the enzymatic activity of delta-6 and delta-5 desaturase, two fundamental enzymes for the metabolism of PUFA, confirm their association with several features of the MetS, also in children.

The enlargement of the sample size of the third study and the implementation of an intervention study with supplements of omega-6 and/or omega-3 PUFA in obese children will help to verify the current results and to better clarify the role of omega-6/omega-3 PUFA and their derived metabolites via CYP450/sEH in BP homeostasis and MetS in children.

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Appendix

1. Beneficial effects of ω-3 PUFA in children on cardiovascular risk factors during childhood and adolescence.

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Review

Beneficial effects of ω-3 PUFA in children on cardiovascular risk factors during childhood and adolescence

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ABSTRACT

Omega-3 polyunsatured fatty acids (ω-3 PUFA) are essential nutrients mainly derived from fish and seafood but present also in vegetables such as nuts and seed-oils. Some epidemiological and clinical studies indicate a protection of ω-3 FA against cardiovascular disease and a favourable effect on cardiovascular risk factors control in adults. The evidences of their effects in children and adolescents are scanty but a possible beneficial role, especially for insulin sensitivity and blood pressure control, has been proposed. In this review we want to focus especially on the evidences, which could justify the assumption of ω-3 in children and adolescents, and to underline the aspects which need further investigation. Mechanisms through which ω-3 FA act are manifolds and still a matter of investigation: beside their interaction with ion channel and their influence on plasma membrane fluidity, probably the main effect is acting as competitor for cytochrome P-450 (CYP) with respect to ω-6 FA. Thus, they can modulate the biosynthesis of eicosanoids and other lipid mediators, which likely exert a protective action. Another suggestive hypothesis is that their beneficial effect is not dependent only on the intake of ω-3 FA, but also on the complex interaction between different nutrients including ω-3 and other FAs with polymorphisms in genes involved in ω-3 FA modulation. This complex interaction has seldom been explored in children and adolescents. Further studies are needed to investigate all these points in order to find a better collocation of ω-3 FA on the available armamentarium for preventive, possibly individualized, medicine.
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1. Biochemical characteristics and dietary sources of ω-3 FA

Fatty acids are a wide family of compounds with important and manifold biological activities. The difference in chain length and saturation status determines their biochemical characteristics and their biological role. Omega-3 (ω-3) fatty acids are a family of long-chain fatty acids containing more than one carbon–carbon double bond (polyunsaturated fatty acids, PUFA). The main members of this family are α-linolenic acid (ALA, C 18:3), eicosapentaenoic acid (EPA, C 20:5) and docosahexaenoic acid (DHA, C 22:6) [1]. ALA is an essential nutrient for mammals because they lack enzymes where to insert the double bond in ω-3 position; the main dietary sources are plants and plant oils, such as soybean oil, flaxseed oil, canola oil and walnuts [2].

In mammals ALA can be converted to EPA, but only in small percentage, and the conversion of EPA to DHA, if any, is very limited [3]. Accordingly EPA and DHA are considered essential fatty acids, deriving mainly from sea-food, especially high-fat cold-water fish, such as salmon, mackerel, herring and trout [2].

Most of the clinical trials carried out to investigate the potential benefits of ω-3 PUFA in adults and in children have used fish oil as supplements of ω-3 PUFA, however in the last few years the attention was focused also on alternative sources of ω-3 fatty acids, like krill oil, vegetable oils, nuts and algae [4,5].

EPA and DHA enter the food chain trough marine phytoplankton, proceeding through marine mammals and fish, which represent the main dietary source for humans [2].

In natural fish oil EPA and DHA are bond in triacylglycerides (TG). Fish oil capsules are concentrates of marine oils, containing 30–90% of EPA and DHA generally bond in ethyl-ester (EE) or re-esterified TG (rTG) [6,7].

Because of the reported health benefits of EPA and DHA, there is an increasing demand for products rich in marine ω-3 PUFA and krill oil is an effective sources of these fats.

Krill are small red-coloured crustaceans (*Euphausia superba*) representing the most dominant members of Antarctic zooplankton [5]. They are rich in long-chain polyunsaturated fatty acids, 40% of which are EPA and DHA, in form of phospholipids (PL). In addition to ω-3 PUFA, krill oil contains carotenoid astaxanthin, a potent antioxidant, vitamins A and E, and other fatty acids [8].

The American Food and Drug Administration has classified krill oil as generally recognized as safe and previous clinical and pre-clinical trials have shown that it is safe and well tolerated [8]. Only a few studies have tested the efficacy of krill oil compared with fish oil and the results go in the direction of a possible stronger effect in raising plasma and red blood cell membrane EPA and DHA [5,7,8].

ALA occurs in vegetable food such as nuts, flaxseeds and vegetable oils like canola oil and soy-bean oil, which represent the main sources of ω-3 in vegetarian diets. However the bioavailability of vegetable ω-3 FA remains a matter of debate and seems to limit their use as supplements [4]. Indeed, most studies using vegetable sources of ω-3 (mainly flaxseed oil) report an increase in plasma and red blood cell membranes content of ALA and partially of EPA but not of DHA [9,10,11,12]. Moreover findings from short and long-term trials with ALA supplements do not show clear evidence of a protective action in cardiovascular risk and therefore the question whether ALA supplements could be important for cardiovascular health remains unanswered [13].

Observational studies and clinical trials suggest a protective effect of nuts consumption on coronary heart disease and some intermediate biomarkers, such as blood cholesterol and blood pressure [14–16]. The beneficial effect of nuts can be mediated through several mechanisms: nuts are rich in PUFA, with a different content of ω-6 and ω-3 in the various types, and in addition they contain fibre, vitamin E, magnesium, potassium and arginine that can contribute to the blood pressure and lipid lowering effect [17].

In the last few years increasing interest was focused on algal DHA-rich oil supplementation: clinical trials reported an increase in plasma and red blood cell DHA content after algal oil supplements, but not of EPA or ALA [4] and, even if in small number, indicate comparable efficacy to fish oil in potential beneficial changes in some markers of cardiovascular risk, in particular on plasma lipid profile [13].

2. Mechanism of action

Ω-3 PUFA exert their biological activities through three main classes of mechanisms: some biological effects depend on their incorporation into cell membranes, other effects derive from a direct interaction with ion channels and other cellular components and, finally, EPA and DHA are the parental compounds of bioactive lipid mediators [2]. The exact mechanisms are not yet completely understood, especially it is not clear whether EPA and DHA share the same pathways or not [18].

Ω-3 PUFA can attenuate the response of T-cells and macrophages through cell surfaces receptors, not yet identified, perhaps by changing the composition of membrane microdomains [19]. A direct interaction with some cellular components can mediate some short-term effects such as the antiarrhythmic effect, which depends on a steric interference with ion channels [20], for example the inhibition of the fast, voltage-dependent sodium and L-type calcium currents [19]. Non-esterified ω-3 PUFA can also directly interact with peroxisome proliferator-activated receptors (PPARs) and others transcription factors, thus modulating gene transcription [18]. Recently a role of GPR120 has been discovered, a member of the family of fatty acid sensing G-protein-coupled receptors (GPCR), mediating the anti-inflammatory and insulin-sensitizing effects of ω-3 PUFA [21].

Other proteins that can be directly activated by AA and ω-3 FA, and consequently probably influenced by their ratio, are protein kinase C, NADPH-oxidase and a two-pore domain K^+ channel [19].

Incorporation of EPA and DHA into cell membranes can modulate the properties of lipid rafts and thus alter the membrane fluidity, affecting hormone receptor binding and the function of membrane-associated proteins [2].

Moreover, EPA and DHA, mostly incorporated into the second position of membranes phospholipids, can be released by phospholipase A2 (PLA2) and converted to a variety of eicosanoids and other lipid mediators through three different metabolic pathways. The first two pathways involve cyclooxygenase (COX) and liopoxygenase (LOX), leading to the formation of prostaglandins, thromboxanes, and leukotrienes; the third branch is catalyzed by cytochrome P 450 (CYP450) leading to the formation of eicosanoids.

Therefore EPA and DHA share the same metabolic pathways of arachidonic acid (AA) and, moreover, it has been shown that they compete with AA for binding with these enzymes, thus inducing profound changes in metabolites biosynthesis that could in part explain the beneficial actions of ω-3 compared to ω-6 FA [18]. The competition of EPA and DHA with AA may determine the synthesis of TXA3, almost inactive, instead of TXA2, which has pro-aggregatory properties. Furthermore, starting from ω-3 FA the metabolism via COX lead to the formation of PGI3 that share the antiaggregatory effect of the AA-derived PGI2. The metabolism of EPA via LOX determine the biosynthesis of LTB5, less potent than the pro-inflammatory AA-derived LTB4.

Moreover, ω-3 PUFA are also the precursors of novel families of compounds, the so-called resolvins, protectins and maresins, with anti-inflammatory and pro-resolving properties [22]. In particular EPA and DHA, through the complex metabolism involving COX-2 and aspirin-dependent formation of intermediate metabolite, followed by a conversion via LOX, are metabolized to the E-series of resolvins, starting from EPA, and to D-series of resolvins,

protectins and maresins from DHA, which counteract the excessive inflammatory while regulating the trafficking of leukocytes and stimulating non-inflammatory phagocytosis of apoptotic neutrophils by macrophages [23].

CYP enzymes accept EPA and DHA as efficient substrates alternative to AA. CYP epoxygenases produce epoxyeicosatrienoic acids (EETs) from AA, epoxyeicosatetraenoic acids (EEQs) from EPA and epoxydocosapentaenoic acids (EDPs) from DHA. CYP hydroxygenases lead to the biosynthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) from AA, 20-hydroxyeicosapentaenoic acid (20-HEPE) from EPA and 22-hydroxydocosahexaenoic acid (22-HDoHe) from DHA [18]. The role of epoxy- and hydroxy-metabolites of AA in cardiovascular function is well known: EETs are mainly involved in antihypertensive and organ-protective mechanisms, in particular they determine vasodilatation in the systemic vascular system, natriuresis in the kidney and act as endothelium-derived hyperpolarizing factors. 20-HETE is involved in both anti- and pro-hypertensive mechanisms, depending on the site of formation and action. It shares with EETs the natriuretic effect but determines vasoconstriction in the vessels [22].

The evidence shows that LOX, COX and CYP enzymes have the ability to metabolize EPA and DHA instead AA, but the relative efficiencies are not well understood. EPA and DHA are generally considered as poor substrates for LOX and COX, compared with AA, whereas they are efficiently metabolized by CYP enzymes with similar or higher rates, compared with AA [18]. A recent study on 20 healthy volunteers indicate that CYP epoxygenases metabolize EPA with an 8.6-fold higher efficiency and DHA with a 2.2-fold higher efficiency than AA, whereas the effects on leukotriene, prostaglandin E, prostacyclin, and thromboxane formation remained rather weak [24]. The evidence of the clinical significance of EPA/DHA metabolism via CYP450 under in vivo conditions is still rare and further investigation is needed.

In animal models, the biological activities of these compounds are partially overlapped to the AA-derived counterparts and partially specific: EEQs and EDPs can be as strong as EETs in vasodilation or even stronger in some vascular beds like cerebral and coronary vessels [25,26]. In an animal model of angiotensin II-dependent hypertension it has been shown a possible role of one EDP isomer as a mediator of the antihypertensive effect of DHA [27]. Some EPA-epoxydes exert anti-inflammatory properties like some EETs regioisomers [28]. Moreover some regioisomers of EEQs and EDPs modify the contractility of neonatal cardiomiocytes, indicating a possible antiarrythmic effect [29].

The suggested hypothesis is that CYP-dependent epoxy-metabolites of EPA and DHA may contribute to the vasodilatory and cardioprotective effects of ω-3 fatty acids and could also serve as biomarkers of EPA/DHA supplementation.

Finally, the results of a recent randomized controlled trial on patients with peripheral vasculopathy suggest another possible mechanism: after 6 months flaxseed oil supplementation, rich in ALA, plasma eicosanoid profile changed with a decrease in soluble epoxide hydrolase products compared with controls and these subjects exhibited a significant decrease in systolic BP. The authors' conclusion is that flaxseed oil may inhibit soluble epoxide hydrolase thus modifying the lipid mediator concentrations that can contribute to the antihypertensive effect [30].

3. Clinical effects of ω-3

Since the late 1960s increasing attention was paid on ω-3 fatty acid, in response to the reports of a lower incidence of coronary heart disease as well as immune and inflammatory disease in Greenland Inuits, whose diet is rich in fish and other sea food [2]. Data from epidemiological and clinical studies have shown that ω-3 fatty acids reduce the incidence of cardiovascular disease, but the mechanisms responsible for this protective effect are not completely elucidated [31,32]. The potential mechanisms proposed are either through its hypotriglyceridemic effect [33,34], a reduced susceptibility to cardiac arrhythmias [35–37], a retardation of atherosclerotic plaque growth [3], an anti-inflammatory effect or a mild hypotensive action [38]. The American Heart Association (AHA) suggests an intake of fish, preferably oily fish, at least twice per week for the general population; in secondary prevention the amount of EPA and DHA should rise to 1 g per day and the supplements should be considered; high doses of ω-3 fatty acids (2–4 g) are recommended in patients needing the triglycerides lowering effect [38].

4. Clinical effects of ω-3 in children

The incidence of obesity and type 2 diabetes reported in children has increased in the last few decades and it has been shown that obesity plays a pivotal role in the development of insulin resistance, which is related to hyperinsulinemia, hypertension, hyperlipidemia, type 2 diabetes and increased risk of atherosclerotic disease. Moreover considerable evidence shows that overweight in childhood and adolescence is associated with insulin resistance, dyslipidemia and high blood pressure in young adults [39].

In the last few years, there has been emerging interest also for the possible beneficial effect of ω-3 in childhood with respect to cardiovascular risk factors.

Some observational studies and a limited number of interventional studies indicate a positive effect of ω-3 supplements on blood pressure control, even if the data currently available are small and not univocal (see Table 1). A recent cross-sectional study on seventy three 8–11-year-old Danish children shows a positive association of mean arterial pressure with whole-blood DHA only in boys and this correlation remains also after adjustment for energy intake, body-fat percentage and physical activity. Ω-3 FA were measured in whole-blood and were found to be associated with fish intake, recorded for 7 days by a Web-based dietary assessment specifically for children [40]. These findings were in agreement with a previous randomized trial of the same group, showing a decrease in systolic blood pressure in healthy 12-months children ($n = 83$), after 3 months of fish oil supplements (mean estimated EPA and DHA assumption: 924 mg/day). Infants were randomly assigned to fish oil supplements or not and to two different milk types and the effect of fish oil supplementation was adjusted for the effects of milk intervention. The fish oil supplements were also inversely associated with plasma triglycerides and directly with total cholesterol and LDL cholesterol [41]. A positive effect on systolic and diastolic blood pressure was found also in adolescent boys aged 13–15 after a 16-week fish oil supplementation (1.5 g EPA and DHA) compared with the control group receiving vegetable oil [42]. In contrast, a cross-sectional study in a hundred and nine 17-year-old adolescents found a poorer metabolic profile, included higher systolic blood pressure levels, associated with a higher DHA content in red blood cell membranes, which remained also after adjustment for physical activity and dietary factors [43].

Data from the National Health and Nutrition Examination Survey indicate that a higher dietary intake of EPA and DHA, recorded by two 24-h dietary recall, in 354 children, aged 8–15 years, born with reduced birth weight, are associated with lower systolic blood pressure and pulse pressure [44]. These findings were consistent with a previous exploratory analysis, which showed a significant inverse association of serum ω-3 PUFA with systolic blood pressure in young adults, aged 24–39 years, born with impaired foetal growth [45]. These data suggest that ω-3 PUFA could play a role in blood pressure control in subjects with low birth weight, which is

Table 1
Studies about the effect of ω-3 FA on blood pressure.

Author, year	Study design and aim	No. subjects	Source and dose	Time period	BP outcome
Damsgaard, 2013 [40]	Cross-sectional study on 8–11 y-o children. To investigate the relationship between whole-blood EPA and DHA and Metabolic Syndrome features	73 (F = 44, M = 29)	Fish intake assessed by a specific paediatric dietary assessment; amount consumed estimated by portion size among 4 different images	–	Positive association of mean arterial pressure with DHA only in boys after adjustment for energy intake, body-fat percentage and physical activity
Damsgaard, 2006 [41]	RCT: 9–12 months infants randomly assigned to fish oil or no supplements and to cow's milk or infant formula. To investigate the effect of fish oil on BP and lipid profile in infants	83 (F = 42; M = 41)	Fish oil (mean estimated EPA and DHA assumption: 924 mg/day)	3 months	Lower SBP (−6.3 mmHg) in infants administered fish oil, also after adjustment for milk intervention
Pedersen, 2010 [42]	RCT on 13–15 y-o boys with a control group receiving vegetable oil. To investigate the effects of fish oil on cardiovascular risk factors	78 (F = 0; M = 78)	Fish oil (1.5 g/day long-chain ω-3 FA)	16 weeks	Lower SBP and DBP (−3.8 and −2.6 mmHg, respectively) in fish oil group
Lauritzen, 2012 [43]	Cross-sectional study on 17 y-o adolescents. To investigate the association between fish intake and Metabolic Syndrome features	109 (M = 44; F = 109)	Fish intake assessed by 7 days food record with pre-coded response categories; intake registered in household measures and portion size based on images	–	Higher DHA status correlated with higher SBP, after adjustment for sex, body fat percentage, dietary factors and physical activity
Skilton, 2013 [44]	Cross-sectional study on 8–15 y-o children born with low birth weight. To investigate the relation between ω-3 FA and BP in children with relative hypertension related to reduced birth weight	354 (F = 174; M = 180)	Fish intake assessed by two 24-h dietary recall, the second after 3–10 days	–	Children in the highest tertile of dietary EPA and DHA intake had significantly lower SBP (−4.9 mm Hg and pulse pressure −7.7 mm Hg) than children in the lowest tertile
O'Sullivan, 2012 [47]	Cross-sectional study on 13–15 y-o adolescents. To investigate the relation between ω-3 FA and BP	814 (F = 395; M = 419)	Three-day diet record measured in household units	–	Inverse association between ω-3 FA and SBP, DBP and mean arterial pressure in boys
Ayer, 2009 [52]	RCT on children in the first 5 years, randomly assigned to fish oil and reduction of ω-6 FA or control (sunola oil). To investigate BP and arterial structure and function in 8 y-o children who received ω-3 FA supplements in the first 5 years of life	410 (F = 203; M = 207)	Canola oil and tuna oil, doses depending on age; every tuna oil capsule had 6% EPA and 27% DHA.	Follow-up at the age of 8 years	No significant differences in BP between intervention group and control group
Van Rossem, 2012 [51]	Observational cohort-study in breast-fed children with a never-breast-fed children control group. To investigate the association between fatty acid composition of infant milk feeding and blood pressure at the age of 12 years	314 (F = 161; M = 153)	Assessment of fatty acid composition of human milk. Infant formula (control) did not contain ω-3 FA	Follow-up at the age of 12 years	Children who received human milk with an n-3 long-chain polyunsaturated fatty acids content above the median had a 4.79-mm Hg lower systolic and a 2.47-mm Hg lower diastolic blood pressure at age 12 years than control
Asserhøj, 2009 [48]	DB-RCT on mother receiving fish oil or olive oil (control). To investigate whether fish oil supplements during lactation affect BP and body composition of children	175 (F = 175; M = 0)	Fish oil (0.6 g/d EPA and 0.8 g/d DHA)	Fish oil supplements in the first 4 months of lactation. Children follow-up at 2.5 years and 7 years	FO boys had 6 mm Hg higher DBP and mean arterial BP than controls, but girls

F, female; M, male; y-o, years old; ω-3 FA, omega-3 fatty acid; RCT, randomized controlled trial; DB-RCT, double blind-randomized controlled trial; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; EPA, epoxyeicosatrienoic acid; DHA, docosahexaenoic acid.

a known factor independently associated with an increased risk of cardiovascular events in adulthood [46].

A cross-sectional study performed on 814 Australian adolescents (13–15-year-old) suggests a possible role of gender in modulating the relationship between ω-3 FA and blood pressure: systolic and diastolic blood pressure were inversely associated with EPA and DHA intake, assessed with a 3-day diet record, in boys but not in girls [47].

Furthermore, an association between blood pressure and the ω-3 content was found in boys but not in girls also in the above mentioned cross-sectional study conducted by Damsgaard [40] and in a trial in breast-fed infants of mother receiving ω-3 supplements, but the latter found an unfavourable relation of fish oil supplements with blood pressure control [48].

Some studies have investigated the long term effect of ω-3 FA supplements administered in early infanthood through lactation or to the mothers during pregnancy but the results are not univocal. The interest stemmed from evidence that breast feeding, rich in EPA and DHA, in contrast to the first formula milk, is associated with lower blood pressure in childhood and adulthood [49,50].

Table 2
Studies about the effect of ω-3 FA on insulin sensitivity.

Author, year	Study design and aim	No. subjects	Source and dose	Time period	Insulin resistance outcome
Burrows, 2011 [60]	Cross-sectional study on 5–12 y-o children. To investigate the relationship between ω-3 Index, weight and insulin resistance	48 (F = 28; M = 20)	Fat intake assessed by a 135-item semi-quantitative food frequency questionnaire	–	A moderate correlation found between ω-3 Index and fasting insulin level and HOMA-IR
Decsi, 2002 [61]	Observational study on 8–16 y-o diabetic children. To compare plasma and red blood cell membranes fatty acids in diabetic children with non-diabetic controls	80 (F = 50; M = 30)	Plasma and red blood cell membranes determination of AA, EPA, DHA and ALA	–	Lower levels of AA and DHA in diabetic children compared with controls
Miller, 2011 [62]	Longitudinal study on 0–8 y-o children with islet autoimmunity. To investigate the correlation between ω-3 FA intake and erythrocyte membrane ω-3 fatty acid levels and type 1 diabetes	167 (F = 82; M = 85)	Dietary intake assessed by a 111-item semi-quantitative food frequency questionnaire	–	No significant association between ω-3 FA and ω-6 FA erythrocyte membranes levels and the onset of type 1 diabetes
Lòpez-Alarcòn, 2011 [63]	RCT on 9–18 y-o overweight and insulin resistant children. To investigate the effect of ω-3 FA supplements on body weight and insulin resistance, compared to placebo group	76 (M; F not specified)	900 mg of ω-3 FA (360 mg DHA + 540 mg EPA)	1 month	Supplementation with ω-3 FA decreased HOMA-IR by 15% after adjusting for puberty, treatment adherence, changes in adipokines, and weight loss
Juàrez-Lòpez, 2013 [55]	Open-label study on 10–12 y-o obese and insulin resistant children assigned to ω-3 FA or Metformin (control). To investigate the effect of ω-3 FA on HOMA-IR and BMI	201 (F = 106; M = 95)	600 mg of ω-3 FA (360 mg of EPA and 240 mg of DHA)	12 weeks	Reduction of glucose and insulin levels while reducing HOMA-IR in ω-3 FA group compared to controls, also after adjustment for weight reduction, sex and age

F, female; M, male; y-o, years old; ω-3 FA, omega-3 fatty acid; RCT, randomized controlled trial; DB-RCT, double blind-randomized controlled trial; HOMA-IR, homeostasis model assessment-estimated insulin resistance index; EPA, epoxyeicosatrienoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; BMI, body mass index.

In an interventional study, children who received human milk with an ω-3 FA content above the median had a nearly 5 mmHg lower systolic and 2.5 mmHg diastolic blood pressure at the age of 12 years compared with never breast-fed children, but ω-3 FA content below the median in the milk was not associated with blood pressure levels at 12 years [51]. In contrast, in a previous trial boys of mother receiving fish oil supplements during the first 4 months of lactation showed a higher diastolic blood pressure at the age of 7 years compared with the olive oil control group [48].

In another study investigating the long-term effects of ω-3 supplements, the supplements of ω-3 FA from infancy to 5 years (canola oil and tuna oil), with a contemporary reduction of omega-6 FA assumption in order to provide an ω-3 to ω-6 ratio of 1:5, do not affect blood pressure and vascular structure at the age of 8 years, even if the concentration of ω-3 FA in the plasma were higher in the intervention group compared with controls [52].

Ω-3 FA supplements are effective in lowering triglyceride levels in adults, therefore in international guidelines their assumption is suggested for patients who need to lower triglycerides. No effects on the other plasma lipid levels such as cholesterol are evident in adults. Little is known about the lipid lowering action of EPA and DHA in children and adolescents, only a small number of clinical trials are available and the results are not always encouraging. In two recent interventional studies in children and adolescents with hypertriglyceridemia ($n = 111$ and $n = 25$, respectively), aged 8–18 and 9–19 years old respectively, 3–6 months fish oil supplements, at the doses of 500–1000 and 3360 mg per day, did not lower plasma triglycerides compared with control group [53,54].

In contrast a positive effect in lowering plasma triglycerides was found in 103 obese children and adolescents after 12 weeks of 1.8 g/day of ω-3 supplements, compared with the control group receiving metformin, and the other plasma lipids were not affected by the treatment [55].

The evidence of an action of ω-3 FA on cholesterol is scanty and not conclusive: Damsgaard found a positive correlation between EPA levels and HDL levels in 8–11 years-old children [40]. However in a previous study fish oil supplements were associated to higher total and LDL cholesterol in 9–12 months infant [41]. In an observational study no association was found between fish consumption, assessed by a 7 day pre-coded food diary, and serum lipid profile in hundred-and-nine adolescents [43].

A large amount of data, from epidemiological studies, indicate a protective effect of ω-3 FA on glucose metabolism and insulin sensibility in adults [56,57]. Accordingly, the results of many clinical trials support the positive effect of ω-3 on glycemic control, however some showed negative results [58,59]. The evidence from observational studies and clinical trials in children is limited but supports a beneficial effect of ω-3 also during childhood (see Table 2).

A cross-sectional study on 5–12 years-old children showed a moderate but significant correlation between fasting insulin levels and HOMA-IR and the ω-3 Index, which is the percentage of EPA and DHA contained in red blood cell membranes, as an index of chronic ω-3 intake [60]. Interestingly, in this population, obese children had altered erythrocyte fatty acid composition unrelated to reported dietary intake and showed lower levels of ω-3 Index compared with non-obese children [60].

Moreover, lower plasma and erythrocyte membrane levels of AA and DHA were found in 40 diabetic children compared with non-diabetic controls [61]. However in a longitudinal study conducted on 167 children at increased genetic risk for type 1 diabetes for the development of persistent islet autoimmunity, ω-3 and ω-6 FA levels in red blood cell membranes were not associated with the development of type I diabetes [62]. In a clinical trial on 76 overweight and insulin resistant children the supplementation with 900 mg per day of ω-3 FA for 1 month decreased fasting insulin

and HOMA-IR, also after adjustment for pubertal status and weight loss [63]. Accordingly, a recent trial showed a reduction of glucose and insulin levels while reducing HOMA-IR after 12 weeks of 1.8 g of ω-3 supplementation in 201 obese and insulin resistant children, also after adjustment for sex, age and change in BMI [55].

There is also increasing interest on the use of ω-3 supplements in the treatment of non-alcoholic fatty liver disease (NAFLD), which is pathogenically linked to insulin resistance and metabolic syndrome. According to the evidence of a positive effect of EPA and DHA supplements on liver steatosis in adults, also the few clinical trials available in children support the hypothesis that DHA can decrease liver fat content in children with NAFLD [64,65].

Animal models show positive results encouraging the use of ω-3 FA to prevent diet-induced obesity [66] and some trials in overweight adults and in obese diabetic women have also reported a beneficial effect in fat mass reduction [67,68]. Nowadays, there is no clear evidence in children and the attention is mainly focused on the possible programming effect of ω-3 fatty acids in breast milk on later infant and young children body composition. The results from the available studies are divergent: some studies show an inverse correlation between maternal ω-3 FA intake and ω-3 FA content in formula milk and later body composition [69–71], others show a direct correlation [72–74], finally in some studies no significant correlation was found [48,75–77]. A recent study on 201 obese children and adolescents indicates a beneficial effect of ω-3 supplements (1.8 g EPA and DHA for 12 weeks), without other lifestyle interventions, on weight reduction [55]. In contrast Nobili reported no effect on BMI after 6 months DHA supplements in children compared with placebo, but the doses of DHA were low (250–500 mg/day) [64].

5. Adverse effects

Omega-3 supplement are generally considered safe and, where reported, the tolerability of ω-3 FA in clinical trials, in children as well in adults, was good with no major adverse reactions. An adverse effect of ω-3 FA supplements is the increased risk of bleeding, due to the potential antithrombotic effect, however the evidence available does not support a clinical relevant increased bleeding, even when ω-3 FA supplements were concomitant with antiplatelet or anticoagulant therapy [78]. Another potential safety concern is the presence of contaminants, especially mercury and dioxins, in fish and fish oil, which has direct implications for dietary recommendation, in particular for some population such as pregnant women and infants [79].

6. Conclusion and perspectives

In summary, the clinical effect of ω-3 FA on cardiovascular risk factors in children is not univocal. Actual evidence supports a beneficial effect of ω-3 FA supplements on insulin sensitivity and a possible positive effect on blood pressure control whereas they are not yet conclusive concerning the effect on plasma lipids and body composition. Anyhow, there were few clinical trials in children and most of the evidence comes from epidemiological studies, sometimes with limited sample size. Moreover, doses of supplements varies largely as well as the duration of the interventions, and it is not easy to detect a dose-response effect among different studies.

In humans, different CYP isoforms are responsible for ω-3 or ω-6 metabolism, whereas other enzymes, such as the soluble epoxide hydrolase (EPHX2), convert these metabolites to mostly inactive compounds. Polymorphisms in these genes have been tested to evaluate their effects especially in blood pressure homeostasis with some encouraging results [80–88] and it is possible that also preferential dietary intake of ω-3 vs. ω-6 could influence their effect. Thus, a suggestive hypothesis is that the beneficial effect of ω-3 FA is not only dependent on their intake and content, but also on the complex interaction between different nutrients and polymorphisms in genes involved in ω-3 FA metabolism [89–97]. These complex interaction has seldom been explored in children and adolescents [95]. Further studies are needed to investigate all these points in order to find a better collocation of ω-3 FA on the available armamentarium for preventive, possibly individualized, medicine.

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Review

Omega-3 fatty acids and cytochrome P450-derived eicosanoids in cardiovascular diseases: Which actions and interactions modulate hemodynamics?

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ABSTRACT

Increasing interest is focused on omega-3 fatty acids (FA) because of their potential beneficial effects, particularly in cardiovascular disease prevention. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two major omega-3 FA, are mainly consumed through diet, particularly from fish and seafood intake, whereas alpha-linolenic acid (ALA) is present in high amounts in leafy green vegetables, nuts and seeds. The hypothesis of a cardiovascular protective action of omega-3 FA derives mainly from observational studies, whereas the evidence from interventional studies is not always consistent. Nonetheless, clinical trials and meta-analyses indicate a positive action, at minimum on blood pressure (BP). Omega-3 FA may act through different biological pathways; however, in our review, we seek to revisit, most notably, the role of their metabolites via cytochrome P450 (CYP450) in hemodynamic modulation. We emphasize that the effect of omega-3 FA may depend on their balance with other dietary compounds, particularly omega-6 FA, which compete for the same pathways, thus modulating the production of metabolites. Furthermore, the biological activity of omega-3 FA might be better explained by the complex balance and interactions between a variety of nutrients and polymorphisms of genes implicated in specific metabolic pathways.

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1. Introduction

Fatty acids (FA) constitute a broad family of compounds, subdivided in different classes on the basis of biochemical structure. The main families with biological and clinical relevance are saturated and *cis*-unsaturated FA, the latter further divided into monounsaturated and polyunsaturated fatty acids according to the number of carbon–carbon double bonds and their *trans*- analogues.

In the last few decades, increasing attention was paid to polyunsaturated FA because of their potential beneficial effects, particularly regarding cardiovascular disease [1].

Whereas the sources of saturated FA are mainly meat and animal products, most polyunsaturated FA derive from vegetable and seed oils. The main sources of omega-6 FA, such as linoleic acid (LA), are vegetable oils, such as safflower oil, sunflower oil and cottonseed oil. Soybean oil and canola oil are contrarily rich in omega-3 FA, notably in alpha-linolenic acid (ALA) [1]. Moreover, nuts are rich in LA and some of them, walnuts in particular, are good sources of ALA [2]. LA and ALA are essential FA whereas eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are long-chain omega-3 FA which may be synthesized from ALA. However, the efficiency of this process in human is generally low. Therefore, they are mainly consumed through diet and occur in seafood as follows: fatty fish, such as salmon, tuna fish and herring, have a high content of these compounds [1].

The mechanisms of action and the biological and clinical effects of omega-3 FA are not completely understood currently and they remain an active area of research.

In this review, we seek to revisit the biological effects of omega-3 FA and those of their metabolites via Cytochrome P450 (CYP450) and explore their possible interactions with other compounds, in particular omega-6 FA. We are interested specifically in probing the effects on blood pressure (BP) and vascular hemodynamics, which may be mediated through this pathway (Fig. 1).

2. Clinical effects of omega-3 FA

Omega-3 FA have a wide range of actions as follows: they may act on the vessels by improving the endothelial function and elastic properties of the arteries [3], exerting a favorable effect on the autonomic system and reducing platelet aggregation [4], exerting anti-arrhythmic action by increasing the arrhythmic thresholds [5] and playing an important role in the modulation of the inflammatory response [6]. Finally, they may improve the serum lipid profile [7]. In summary, the rising interest in Omega-3 FA is due to their pleiotrophic effects and to the possible eventual protective effect on cardiovascular disease, although the available data are not always consistent. In fact, despite observational studies that have mostly shown a protective effect, the results from randomized controlled trials (RCT) have not always been uniform and successive meta-analyses have questioned the presence of a large omega-3-FA effect in at-risk populations.

For example, a recent large prospective study conducted on healthy older adults investigated the role of omega-3 FA consumed through the typical diet in primary prevention. The results showed that plasma omega-3 FA, considered as single FA (EPA, DHA and DPA) and total Omega-3 FA, were associated with lower total mortality, largely dependent on fewer cardiovascular deaths and in particular fewer arrhythmic deaths [8]. Moreover, total mortality was inversely associated with EPA and DHA in a linear fashion, whereas the inverse association with total omega-3 FA was not linear, in accord with previous studies [9]. However, it should be considered that circulating FA are influenced by short-term fluctuations in dietary FA consumption, which probably affects the true relationship between plasma FA and mortality.

Additionally, some large interventional trials supported the beneficial effect of omega-3 FA in cardiovascular disease as follows: in the GISSI-Prevenzione trial, the treatment by omega-3 FA lowered the risks of death, non-fatal myocardial infarction and stroke in a sample of 11,324 patients surviving a recent myocardial infarction [10]. Another trial, investigating the effect of EPA supplementation in primary prevention in 14,981 hypercholesterolemic subjects on statin therapy, indicated a reduction in the incidence of coronary artery disease, particularly in the patients with a high-risk dyslipidemic pattern (high triglyceride level and low HDL-cholesterol level) [11]. Regardless, recent meta-analyses, including a large number of primary studies, have not demonstrated unequivocal results concerning cardiovascular risk [12–14]. In a systematic review and meta-analysis, including 20 studies with a total of 68,680 patients, omega-3 FA supplements were not associated with a lower risk of all-cause mortality, cardiac or sudden death, myocardial infarction or stroke [12]. Another meta-analysis, including 14 randomized placebo-controlled trials involving 20,485 patients with a history of cardiovascular disease, reported insufficient evidence of a beneficial effect of omega-3 FA supplements in secondary cardiovascular prevention [14]. However, it is worth considering that the trials included in the meta-analysis had a short follow-up period (2 years or less) and that the above-mentioned large trials [10,11] with positive findings were excluded from some meta-analyses because they were not placebo-controlled, suggesting a cautious interpretation of the results. The most recent meta-analysis on the same topics arrived at the same conclusion when considering the association between coronary risk and fatty acids from dietary intake, assessed by questionnaire or using dietary records, or by circulating levels of fatty acids. Comparing the participants in the top third to those in the bottom third, dietary long-chain omega-3 FA and plasma levels of EPA and DHA were associated with a nearly 13% lower coronary risk [15]. When assessing the effect of omega-3 FA supplements in RCT, no significant reduction in coronary risk was found [15].

Uncertainty also remains regarding the relationship between fish or omega-3 FA consumption and cerebrovascular disease. A systematic review and meta-analysis of 26 prospective cohort studies and 12 RCT, which investigated the relation between the risk of cerebrovascular disease and omega-3 FA and fish consumption in primary and secondary prevention, showed the presence of a moderate, inverse association [16]. The analysis of the prospective trials indicated a protective effect of fish intake with regard to the risk of cerebrovascular disease in the general population and showed that an increment of fish intake (at least 2 servings per week) reduced cerebrovascular risk by 4%. Interestingly, the analysis of the different type of fish consumed showed a possible favorable effect of fatty fish, whereas such findings were not significant for white fish. Conversely, the RCT analysis of omega-3 FA supplementation did not indicate any significant correlation with cerebrovascular risk [16].

The evidence concerning the beneficial effect of EPA/DHA on BP appears clear as follows: many interventional studies with fish oil have suggested a beneficial effect of omega-3 supplements on BP control, particularly in hypertensive subjects, with a possible threshold effect. The first meta-analysis to show a positive antihypertensive action of omega-3 FA in hypertensive patients, compared to normotensive subjects, was reported by Morris et al. in 1993 [17].

These results were consistent with a subsequent meta-analysis of 36 studies evaluating a total sample of 2114 subjects receiving high doses of fish oil (mean dose 4.1 g/day) as follows: fish oil significantly reduced BP (SBP–2.1 mmHg and DBP–1.6 mmHg), with a stronger effect in hypertensive and older (>45 years) subjects. Interestingly, no dose-response relationship was observed and the effect of low doses (<500 mg/day) of omega-3 supplements

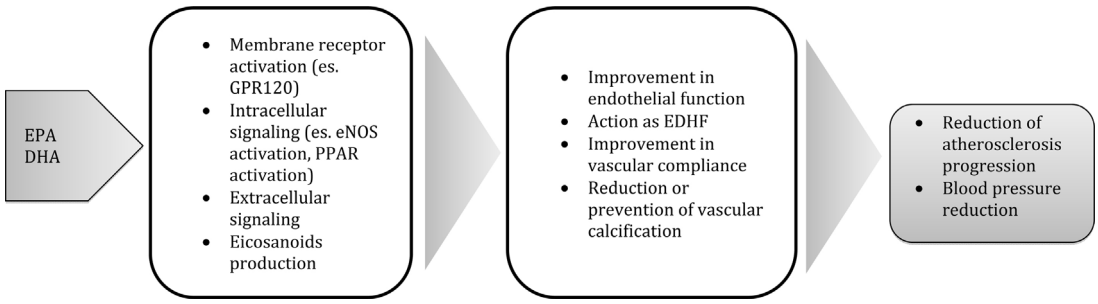

Fig. 1. Our hypothesis: omega-3 FA may positively affect the vascular function determining blood pressure reduction.
In particular, omega-3 FA act as vasodilators, improve endothelial function and reduce arterial stiffness and calcification in part through their incorporation into cell membranes and extra- and intra-cellular signaling and partly through their CYP/sEH metabolites. EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; eNOS: endothelial Nitric Oxide Synthase; PPAR: Peroxisome proliferator-activated receptor; GPR120: G protein-coupled receptor 120; EDHF: Endothelium-Derived Hyperpolarizing Factor.

remains unclear [18]. They observed a dose-response effect when the studies were grouped by Omega-3 FA dose; however, it should be emphasized that the omega-3 dose was relatively high in the group receiving the lowest dose (up to 3 g/day).

The most recent meta-analysis examined 70 randomized controlled trials with EPA + DHA supplements in hypertensive and normotensive subjects [19]. The mean dose of EPA and DHA was 3.8 g/day, deriving mostly from fish oil and also from EPA- and DHA-fortified foods, seafood and algal oil. Omega-3 supplements provided a reduction in systolic BP (SBP) of 1.52 mmHg and diastolic BP (DBP) of 0.99 mmHg in the meta-analysis of all studies with hypertensive and normotensive subjects, compared with placebo (mostly olive oil and other vegetable oils). The analysis of untreated hypertensive subjects showed the strongest effect for EPA + DHA in lowering BP, compared to normotensive subjects [19].

Prospective cohort studies have also examined the impact of the omega-3 FA dietary content on the development of hypertension in normotensive subjects, with some finding an inverse association [20,21] and others no association [22,23]. A meta-analysis, including 8 of these studies with approximately 56,000 subjects followed up for 3–20 years, showed that the subjects with the highest dietary consumption of omega-3 FA, as determined by plasma or erythrocyte fatty acid content, had a lower risk of developing hypertension compared to subjects with the lowest intake [24]. Interestingly, this finding supports the hypothesis of a stronger protective effect for DHA in primary prevention.

A recent study on 312 subjects, evaluating the impact on BP of lower doses of EPA + DHA (0.7 and 1.8 g/day, respectively), which are more easily achievable through dietary modification, showed that only patients with isolated systolic hypertension exhibited a significant reduction in SBP (- 5 mmHg) [25].

Current guidelines by the American Heart Association Nutrition Committee suggest the consumption of at least 2 servings of fish per week for primary cardiovascular prevention and Omega-3 supplements for secondary prevention [26,27]. Regarding BP, the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC) guidelines published in 2013 recommend that patients with hypertension eat fish at least twice a week [28], without specifying the type of fish, whereas the 7th and 8th Joint National Project reports mention neither fish nor omega-3 FA [29,30].

Recently, increasing attention was directed to the possible protective effect of omega-3 FA on the development of hypertension in children, who might comprise an interesting population because of the lack of many confounders present in adults. Thus, some observational studies and a few interventional studies support a positive effect of omega-3 FA on BP control in childhood as well [31–33].

In addition to the actions of EPA and DHA, recent studies found a BP-lowering effect of flaxseed oil, which is rich in ALA as follows: in a double-blind, placebo-controlled RCT (FlexPAD trial) in 110 patients with peripheral artery disease (PAD), the treated group (30 g/day of flaxseed) showed an increase in plasma ALA, which was inversely associated with BP, and they exhibited a decrease in SBP and DBP after 6 months that was significant in hypertensive patients [34]. A subsequent study on the same population in the FlexPAD trial moreover displayed a reduction in central SBP and DBP after flaxseed supplementation [35].

The possible stronger effect of dietary fish on BP, compared to EPA/DHA supplementation, raises some unanswered questions. First, it warrants consideration that the biological role of single nutrients probably cannot be separated from the wide range of other substances contained in a food. For example, fish is rich in vitamins [36], amino acids [37] and some trace elements [38] that may also exert favorable vascular effects. Second, the intake of certain foods should be assessed in the context of the whole diet, considering the balance between the different nutrients that may exert a variety of actions and interactions.

2.1. Vascular effects of omega-3 FA

A clinically relevant action of omega-3 FA is the BP-lowering effect, which is believed to derive from reduced systemic vascular resistance and improved endothelial function, thus interfering with the atherosclerotic process [39]. The modulation of vascular functions remains, in our opinion, one of the key aspects of cardiovascular protection by omega-3 FA.

Studies on animal models suggest that EPA, but probably not DHA and DPA, may induce Ca++-independent activation and translocation of endothelial nitric oxide synthase (eNOS) with consequent endothelium-dependent vasodilation [40]. In vivo, EPA induces an increase in eNOS phosphorylation via the up-regulation of uncoupling protein-2 (UCP-2) and activation of AMP-activated protein kinase [41]. In the past few decades, dozens of studies in humans were conducted with divergent results concerning the effect of omega-3 FA on endothelium-dependent vasodilation, whereas no effect was consistently observed regarding omega-3 FA supplements on endothelium-independent dilation. Two meta-analysis, including 16 clinical trials, show improvement in endothelial function after omega-3 FA supplementation [42,43]. However, this result warrants a careful evaluation as follows: first, in the meta-analysis conducted by Wang and colleagues, in addition to trials with EPA + DHA, studies with ALA supplements were also included, whereas Xin and colleagues considered only EPA + DHA supplementation for the analysis. Moreover, the sample size in most of the primary studies was low, and the significance of the

results appeared mainly dependent on the contribution of the low-quality studies [44,45]. Regardless, both meta-analyses included a high-quality randomized double-blind trial in 312 healthy non-smoking subjects with increasing doses of EPA + DHA (0.45, 0.9 and 1.8 g/day) [46]. Compliance with the intervention was verified by a significant increase in the EPA and DHA content of red blood cell membranes; however, endothelial function, as measured by Flow Mediated Dilation (FMD), was not related to EPA + DHA intake [46].

Thus, further studies and large-scale RCT remain required to define the role of omega-3 FA on endothelial function. Moreover, the evidence concerning the effect of EPA and/or DHA on arterial stiffness suggests a possible protective effect, though the clinical significance remains to be clarified. The largest observational study was conducted in a subcohort of the Framingham study, including 3055 subjects as follows: a moderate association between red blood cell omega-3 FA content and several measures of arterial stiffness was observed, in particular, the carotid-femoral pulse wave velocity, which is considered the gold standard index of aortic stiffness. However, after multivariable adjustment, only a modest correlation between higher omega-3 content in red blood cell membranes and lower aortic stiffness remained [47]. A recent small interventional study on 29 subjects receiving EPA + DHA supplements (2 g/day) reported a significant improvement not only in flow-mediated dilation but also in pulse wave velocity, a measure of aortic stiffness [48]. Despite the scarce and mostly underpowered RCT in humans, a recent meta-analysis appears to confirm the association between omega-3 FA supplements and arterial stiffness and arterial compliance; interestingly, the results were not affected by BP changes, indicating a possible BP-independent effect of omega-3 FA on arterial function [49].

Moreover, epidemiological and experimental studies suggest that EPA and DHA are associated with less vascular calcification, probably due to their preventive effect on tissue remodeling [50–52].

A population-based observational study on 1570 subjects in the Netherlands investigated the correlation between fish and EPA + DHA intake, assessed using a 170-item semiquantitative food-frequency questionnaire and coronary calcification measures determined by CT scanning according to Agatston's method. The investigators found that subjects with a higher fish intake had a significantly lower prevalence of mild to moderate coronary calcification compared to subjects who did not consume fish, whereas EPA + DHA intake showed no significant association [53]. No interventional trials on humans are available to date to determine the effect of EPA and DHA on vascular calcifications.

3. Mechanisms of action of omega-3 FA

EPA and DHA exert their biological effects mainly through three classes of mechanisms, although their relative importance concerning the cardiovascular effects is not yet completely understood as follows: first, they may directly interact with ion channels and other cellular components; second, they may be incorporated into cell membranes; and finally, the factor that we consider the most mechanistically relevant, at least for BP homeostasis, is that they are the precursors of a wide family of bioactive lipid mediators [1]. Free omega-3 FA may interfere directly with ion channels without incorporation into cell membranes, thus modulating different actions such as anti-arrhythmic effects [54]. When incorporated into cell membranes, they alter the phospholipid composition, thus modifying the properties of lipid rafts and caveolae and contributing to membrane fluidity [1,6,55]. The incorporation into membrane phospholipids affects the functions of hormone-receptor and membrane-associated proteins as follows: the activation of the pro-inflammatory transcription factor nuclear factor-kappa B is inhibited and the signaling pathway via G protein-coupled receptor GPR120 is activated [6].

However, as the principal mechanism, omega-3 FA, and in particular EPA and DHA, are the precursors of a substantial number of bioactive compounds through the following three main metabolic pathways: the cyclo-oxygenase (COX) pathway, the lipoxygenase (LOX) pathway and the so-called third branch via Cytochrome P450 (CYP450) [56]. These metabolic chains also metabolize arachidonic acid (AA), an omega-6 FA, which competes with EPA and DHA for the binding with these enzymes [57,58].

3.1. CYP450-derived metabolites of AA

The metabolites of AA generated through the three pathways and their clinical and biological effects are better understood compared to those derived from EPA and DHA. In this paragraph, we briefly review the evidence concerning the AA-derived metabolites via CYP450, which have well known hemodynamic effects and, notably, may interfere with the formation of the eicosanoids of EPA and DHA.

COX and LOX metabolize AA leading to the production of prostaglandins, prostacyclin, thromboxane and leukotrienes, which are involved in the modulation of pulmonary and renal function, and in vascular tone and inflammation [56]. Less attention was directed to the third metabolic pathway, the cascade leading to the formation of CYP-derived AA metabolites. In fact, CYP epoxygenase catalyzes the formation of epoxyeicosatrienoic acids (EETs), whereas CYP hydroxygenase leads to the biosynthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) [59]. EETs are generally involved in protective mechanisms and exert an antihypertensive effect as follows: in most vascular beds, they act mainly as endothelium-derived hyperpolarizing factor (EDHF) and may also activate eNOS, leading to a vasodilatory effect [60]. Moreover, they exert a Na+-excreting action [56]. At the renal level, 20-HETE shares the Na+-excreting effect with EETs; conversely, in the vascular system, 20-HETE constricts renal, cerebral, mesenteric and skeletal muscle arterioles [56]. Consequently, 20-HETE acts in opposite directions, eliciting both pro- and anti-hypertensive effects.

Furthermore, EETs mediate anti-inflammatory actions [61] and their less potent metabolites dihydroxyeicosatrienoic acids (DHETs), produced by the soluble Epoxide-Hydrolase (sEH), have antithrombotic effects and may be involved in the inflammatory process, with some reports suggesting them to be pro-inflammatory [62]. Whereas a number of studies in animal models recognized a role of 20-HETE and/or EETs in the development of hypertension [63,64], to date a small number of human studies are available. In renovascular hypertension in particular, some clues were unveiled regarding the involvement of CYP-derived metabolites of AA, notably 20-HETE [65]. Two studies indicated the involvement of CYP-derived metabolites of AA in the regulation of the maternal circulation during pregnancy and a possible contribution to pre-eclampsia, although their pathophysiological role is not defined to date [66,67].

Previous studies in animal models showed the possible modulation of BP by EETs in pregnancy [68,69], some of them suggesting instead their protective role against pregnancy-induced hypertension [69]. In pregnant women, high levels of EETs were found in the fetoplacental circulation compared to that of the maternal circulation [67] and within intrauterine tissues [70,71,69] and some studies support the hypothesis that EETs play a role in BP regulation in pre-eclamptic pregnancies [66,68,72].

Additionally, genetic studies provided some clues concerning the possible role of these compounds in BP regulation and cardiovascular risk. In fact, human genes encoding for the major CYP

isoforms codifying for the enzymes that form EETs along with *EPHX2*, the sEH gene, are highly polymorphic and a number of studies have shown that some variants are associated with a higher risk of hypertension, stroke or other major cardiovascular endpoints [73] with a possible gender-specific effect [74,75].

Thus, a suggestive hypothesis is that omega-3 FA may additionally exert their action through the complex interaction with polymorphisms in genes encoding for CYP enzymes as was similarly hypothesized in a recent study, in which a stronger overtime reduction in BP was significantly associated with a higher ω-3 PUFA intake, but only in subjects carrying the *CYP4F2 433VV* genotype [76]. In fact, the analyzed genotype did not show an association with BP by itself in the whole population; but a significant correlation was found only when considering the interaction with ω-3 PUFA intake, thus suggesting that ω-3 PUFA can exert their protective effect on BP only in people carrying selected genotypes.

3.2. CYP450-derived metabolites of EPA and DHA

As previously stated, not only AA but also EPA and DHA are metabolized by COX and LOX, but particularly by CYP450 leading to the biosynthesis of a wide range of compounds that are currently attracting active research. The EPA-derived counterparts of EETs through the metabolism of CYP-epoxygenase are epoxyeicosatetraenoic acids (EEQs), whereas the DHA-derived counterparts are epoxydocosapentaenoic acids (EDPs). CYP-hydroxylase converts EPA to 19- and 20-hydroxyeicosapentaenoic acid (19- and 20-HEPE) and DHA to 22-hydroxydocosahexaenoic acid (22-HDoHe), which are the counterparts of 20-HETE [77] (Fig. 2).

Epoxy metabolites of AA, EPA and DHA are further metabolized by the sEH, generating dihydroxy-fatty acids, or diols, which are the DHETs dihydroxyeicosatetraenoic acids (DiHETE) and dihydroxydocosapentaenoic acids (DHDP), respectively. This metabolic step is moreover the object of competition between AA and DHA/EPA for enzymatic binding [78].

The biological properties of the epoxides and diols derived from EPA and DHA are not yet completely understood, although substantial interest has been raised recently. In fact, the EPA and DHA epoxides have at least similar but often stronger effects than EETs, in particular concerning their vasodilator [79],anti-inflammatory [78,80,81] and analgesic actions [78].

Animal models using canine and porcine coronary microvessels [79,82] and in rat cerebral artery [83] support the hypothesis that EEQs and EDPs act as endothelium-derived hyperpolarizing factor (EDHF) by activating Ca++-activated K+ channels and that they have a greater vasodilatory action with respect to EETs, which might be the mechanism, or at least one of the mechanisms, responsible for the BP-lowering effect of omega-3 PUFA.

Other studies in animal models stress the role of omega-3 epoxides in BP control as follows: in Angiotensin II-dependent hypertensive mice, an omega-3 rich diet in combination with the sEH inhibitor lowered BP, suggesting that omega-3 epoxides contribute to BP lowering [84]. Moreover, the same group focused attention on DHA-derived epoxides and provided some particular clues regarding 19,20-EDP as a mediator of the anti-hypertensive effect of DHA [85]. Additionally, in CYP1A1 knockout mice, the involvement of 17,18-EEQ and 19,20-EDP in BP control has been shown, suggesting vasodilation via increases in nitric oxide as a pathophysiological mechanism [86].

Moreover, the CYP/sEH-derived metabolites of omega 3 PUFA are involved in angiogenesis regulation, at minimum in retinal and tumoral vascularization as follows: omega-3 epoxide have anti-angiogenic properties [87–89], whereas sEH-derived diols may exert pro-angiogenic action [90]. Contrarily, several studies on animal models revealed that EETs were linked to angiogenesis [91–93].

Finally, EPA and DHA epoxides, along with EETs, exert anti-inflammatory actions [78,80,94]. Thus, our hypothesis maintains that the cardioprotective effects of Omega-3 FA may be explained, at least partially, by the replacement of AA-derived EETs by the more effective EPA- and DHA-derived EEQs and EDPs.

Because no reports are available concerning the biological role of 20-HEPE and 22-HDoHE in hemodynamic modulation in humans, the question of whether they share or perhaps antagonize the vasoconstrictor action of 20-HETE remains unanswered.

4. Metabolic interactions between omega-3 and omega-6 PUFA

The wide range of the metabolic pathways and biological effects of omega-3 FA makes it difficult to completely understand the clinical role of omega-3 FA and their metabolites, which remain a matter of debate. The overall picture is complicated by the possible interactions with other nutrients and dietary compounds that are far from being completely elucidated.

First, consideration should be given to the competition of omega-3 FA with omega-6 FA for the binding with several enzymes. ALA competes with LA for metabolism by delta-6 desaturase and the subsequent enzymes of the pathway, leading to the formation of EPA and AA, respectively [1]. The COX pathway catalyzes the production of prostanoids, such as the pro-thrombotic TXA2 and the anti-thrombotic PGI2, starting from AA. The counterparts derived from EPA exert more potent antiaggregatory (TXA3 and PGI3) and weaker pro-inflammatory actions (LTB5) [59]. The anti-inflammatory effect of omega-3 is further explained by a novel family of compounds, the so-called protectins, resolvins and maresins [95].

Indeed, previous studies have demonstrated that many isoforms of CYP enzymes convert EPA and DHA with equal or even higher metabolic capacities compared to AA and notably, they show largely different regioselectivity [77]. In animal models, EPA and DHA have been demonstrated to replace AA in membrane phospholipids and concomitantly, clear modification exists in the balance of their metabolites with a tissue-specific effect. In rats fed with EPA + DHA supplements, the endogenous formation of EETs, EEQs and EDPs was shifted in favor of EPA and DHA metabolites with different rates in the various tissues, e.g., in the heart, the EET: EEQ: EDP ratio shifted from 87:0:13 to 27:18:55, whereas the modification was in the same direction but with different rates in other tissues, like kidney and liver; only in brain the changes were slight [96]. Additionally, 20-HETE decreased after EPA + DHA supplementation with a concomitant increase in 20-HEPE and 22-HDoHE [96]. An interventional study in humans tested the effect of EPA + DHA supplements on the Omega-3 Index and on the balance of metabolite production. The results were consistent with the previous study on rats as follows: after increasing doses of omega-3 FA supplements for 8 weeks in 20 healthy volunteers, the Omega-3 Index increased in a time- and dose-dependent manner and a large increase was also observed in the EPA-derived metabolites via CYP epoxygenase. In particular, the ratio between the metabolites and precursors of fatty acids indicated that CYP epoxygenase was 8.6 times more efficient in EPA metabolism and 2.2 times more efficient in DHA metabolism compared to AA. Regarding the CYP-hydroxygenase pathway, no modification in 20-HETE and 22-HDoHE were observed, whereas the concentration of 20-HEPE increased 3-fold. Notably, the effect on COX and LOX remained rather weak with respect to that on the CYP epoxygenases, indicating that CYP-dependent metabolites of EPA and DHA are the putative mediators of the cardiovascular protective effects of omega-3 FA [97].

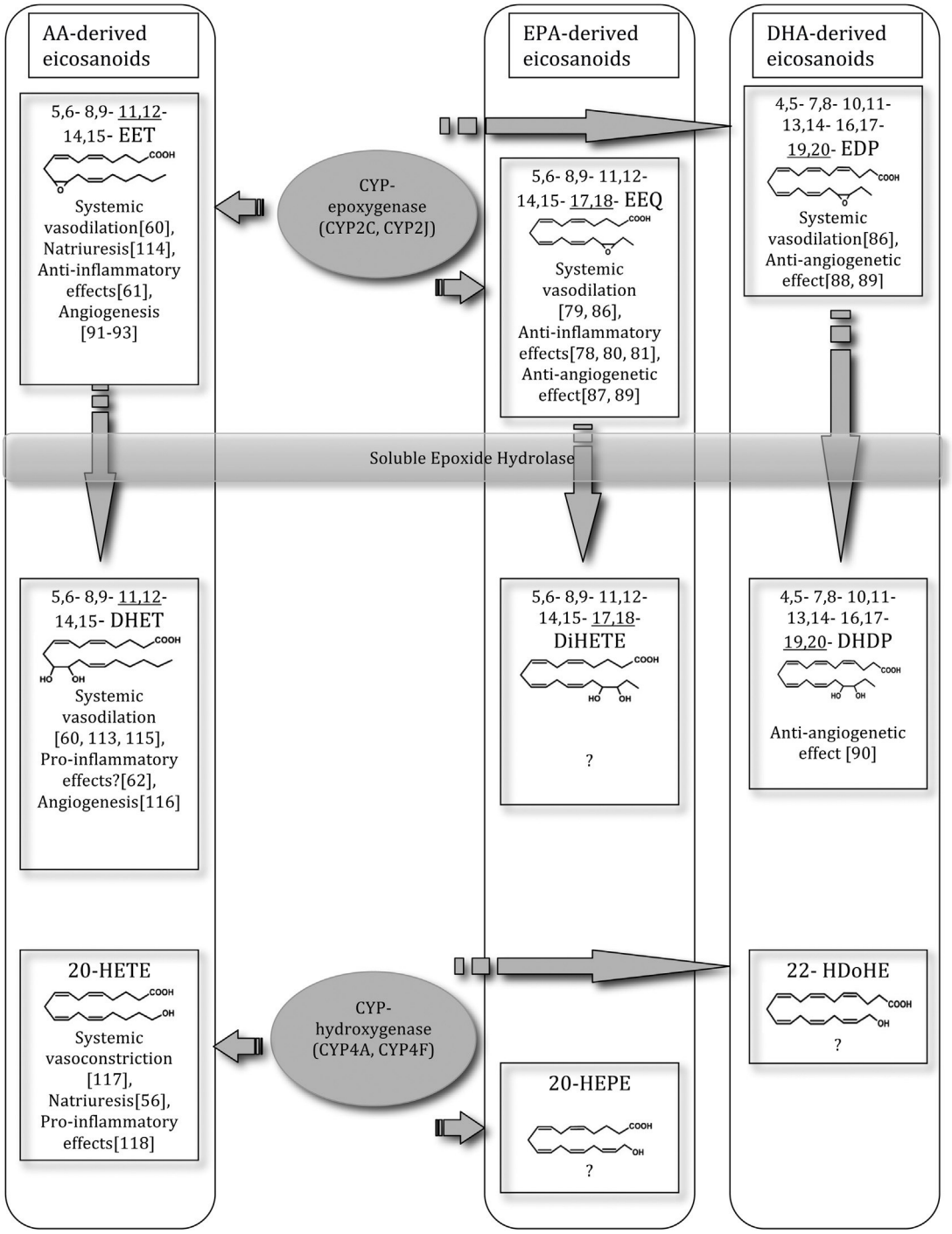

Fig. 2. Main AA, EPA and DHA metabolites via CYP450/sEH and their principal actions [113–118].
The metabolites of AA, EPA and DHA are constituted by different isomers, which are listed in the figure; the illustrated structure refers to the underlined isomer.
AA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; EETs: Epoxyeicosatrienoic acids; 20-HETE: hydroxyeicosatetraenoic acid; EEQs: epoxyeicosatetraenoic acids; 20-HEPE: 20-hydroxyeicosapentaenoic acids; EDPs: epoxydocosapentaenoic acids; DHET: dihydroxyeicosatrienoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DHDP: dihydroxydocosapentaenoic acid; 22-HDoHE: 22-hydroxydocosahexaenoic acid.

5. Possible confounders when evaluating the clinical effect of omega-3 FA

Notwithstanding the understanding of the biological mechanisms of action of EPA, DHA and their metabolites, uncertainty remains about their clinical efficacy. In particular, as stated previously, a discrepancy between observational and interventional studies is often evident. A possible confounder is the use of vegetables oils as controls, and often olive oil, which may in turn exert protective actions [98–100], thus partially blurring the positive effect of omega-3 FA.

Moreover, the protective effect was reported in trials using fish as the source of omega-3 FA compared to EPA + DHA supplements, although supplementation may easily provide higher doses of omega-3 FA than those supplied by fish in the diet. In our opinion, further investigation is warranted to elucidate this complex issue, that is, whether a favorable effect of omega-3 FA should be traced to these dietary fatty acids alone or to the overall sources of these com-

pounds. Fish in particular is rich in many macro and micronutrients [101], such as vitamin D, branched chain amino acids, potassium and magnesium, that may act independently or even synergistically with omega-3 FA [102–104]. Furthermore, the intake of each individual nutrient should be considered in assessing the whole dietary pattern as follows: the intake of fish may be generally assumed to indicate healthier dietary habits and in particular, higher fish consumption is usually associated with higher vegetable and fruit intake and lower intake of meat, which are altogether often related to a higher socioeconomical status [105,106].Thus, the effects of Omega-3 FA may indeed derive from the interaction with other nutrients, such as fiber, olive oil or antioxidants, which are considered to play a role in cardiovascular risk reduction [107]. Indeed, some recent studies and meta-analyses have called attention to the role of dietary models rather than single nutrients with respect to cardiovascular risk. A systematic review of prospective studies or RCT investigating the relationship between dietary exposure and coronary heart disease identified vegetables, nuts, fish and marine Omega-3 FA, fruit, fiber and the "Mediterranean diet" as protective factors, whereas *trans* fatty acids and foods with high glycemic indexes were recognized as harmful factors [108,110]. A recent RCT showed that a Mediterranean diet supplemented with olive oil or nuts, which are rich in omega-6, reduced the incidence of major cardiovascular events (myocardial infarction, stroke or death from cardiovascular causes) in primary prevention [109].

In our opinion, greater attention should be directed additionally to the balance between the different types of fatty acids. Undeniably, consideration should be given to the profound change in dietary patterns during the last century in Western countries whereby the omega-6: omega-3 FA balance shifted from a ratio of 1–2:1 in the Paleolithic era to the current 15–20:1 ratio [111]. This unbalance and the changes in the dietary intake of vegetables, fiber, nuts and berries are considered key factors in the development of cardiovascular disease.

Finally, the response to omega-3 FA supplements, in terms of modifications of their metabolite profile, appears to show a high grade of inter-individual variability [112], which may consequently obscure the clinical effect of omega-3 supplementation in large clinical trials.

6. Conclusion

In conclusion, evidence from observational studies, in particular, support the recommendation to enhance the intake of omega-3 FA, primarily from seafood, to reduce cardiovascular risk. We believe that their protective effects are probably mediated by an improvement in vascular function with a consequent antihypertensive effect due to the shift of CYP-derived metabolites to more potent vasodilatory agents. Persistent debate remains concerning the best source of omega-3 FA and whether the beneficial effects may be explained only by their specific biological actions or rather by their complex balance and interactions with a variety of nutrients and polymorphisms of genes implicated in their metabolic pathways.

Conflicts of interest

None.

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